GASTRIN AND GASTRIN RECEPTORS IN COLORECTAL NEOPLASIA

by

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SUMMARY

Colorectal cancer remains a common public health issue and a leading cause of mortality in the industrialised world. Much still needs to be learned about the factors regulating the proliferation of colonic epithelial cells but a large body of work suggests that the gastric antral hormone gastrin may be a trophic factor for colorectal cancer cells. This putative role for gastrin is controversial, however, and several questions remain unanswered. The aim of this thesis was to address several of these questions and investigate further the possible involvement of gastrin in colorectal neoplasia.

In the first study (Chapter 9), the effects of omeprazole-induced endogenous hypergastrinaemia on the subsequent development of azoxymethane-induced colorectal neoplasia in rats was examined. Omeprazole was chosen because of the well documented elevations in circulating gastrin concentrations seen during therapy with this commonly prescribed drug. The azoxymethane model was chosen because of its similarities to human carcinogenesis and because it has been well characterised and widely used in recent years. Despite having marked hypergastrinaemia throughout the study, significantly fewer rats in the omeprazole group developed tumours and had fewer tumours per rat. The reasons for this are unclear but omeprazole is a potent inducer of the cytochrome P450 enzymes responsible for the metabolism of numerous carcinogens. It is possible that omeprazole treatment altered hepatic and/or intestinal azoxymethane metabolism, rendering it less effective as a colonic carcinogen. Future experiments are merited to investigate further the results of this study and these are discussed in Chapter 13.

The second study (Chapter 10) examined the unresolved issue of whether plasma gastrin levels are elevated in colorectal tumour patients relative to patients without tumours. The study controlled for all known causes of hypergastrinaemia and measured both fasting and meal-stimulated plasma gastrin concentrations pre- and postoperatively. When studied in this manner, gastrin levels were similar in both tumour and control patients. In all but one case very high gastrins occurred in patients with positive gastric autoantibodies and were likely to result from undiagnosed atrophic

gastritis or pernicious anaemia. Furthermore, no fall in gastrin concentrations was seen following presumed curative resection. It was notable that five tumour patients with perioperative loss of *Helicobacter pylori* (HP) infection had reductions in postoperative plasma gastrin. The results of previous studies should be reconsidered in the light of the important effects of HP infection on plasma gastrin. The results of this study have shown conclusively that colorectal tumour patients do not have higher circulating gastrin concentrations than well-matched controls.

Next (Chapter 11), the content of gastrin and its processing-intermediates in samples of colonic carcinomas and disease-free mucosa was determined in a study aimed at assessing a possible role for gastrin as an autocrine growth factor in this malignancy. The study demonstrated the practical difficulties encountered in accurately measuring peptide levels in extracts of heterogeneous tissues. Once early technical problems of non-specific interference were resolved, the results showed that significant amounts of both bioactive, carboxyamidated gastrin and its processing-intermediates were present in both normal and malignant colon. Levels were similar in the two tissues although the results of trypsinisation suggest that tumours contain markedly more progastrin than corresponding normal mucosa. This is compatible with defective post-translational processing of gastrin in tumour cells although the lack of an antibody specific for progastrin itself prevents a definitive answer on this point. As peptide concentrations were similar in both tissues it is unlikely that synthesis of gastrin peptides is a novel feature restricted to neoplastic colorectal epithelium. It does not detract, however, from the hypothesis that gastrin produced locally may be a relevant trophic factor for tumour cells at this site.

The final study (Chapter 12) explored further the possible presence of gastrin/CCK-B receptors in membranes preparations made from human colorectal cancers. The well characterised cell line AR42J was used as a 'positive control' to establish whether high affinity gastrin/CCK-B receptors could be detected by radioligand binding. This proved to be the case and no loss of binding was noted in crude membranes prepared from the cells. When a similar assay was applied to membranes from normal and malignant human colon, no convincing evidence for high

affinity receptors was found although small amounts of specific binding of uncertain significance were observed. The possible reasons for the negative results are discussed as are alternative methods of studying these receptors. This issue is an important one to resolve before the increasing number of highly selective and potent gastrin/CCK-B receptor antagonists can be considered as possible therapeutic options for patients with colorectal cancer.

In conclusion, the studies presented in this thesis have examined the role of gastrin in several ways. No convincing evidence supporting a simple trophic role in colorectal neoplasia was found. If such a relationship exists it is likely to be more subtle and complex than hitherto supposed. Further studies focusing on the autocrine/paracrine effects of gastrin peptides (especially progastrin and glycine-extended intermediates) are required and advantage needs to be taken of the recent cloning and sequencing of the gastrin/CCK-B receptor genes. Together these approaches may define more closely what relevance, if any, gastrin has for tumours arising from the colon and rectum.

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DECLARATION

I declare that the work contained in this thesis is original and has not previously been submitted for consideration of a Higher Degree.

The experimental work for this thesis was carried out while I was employed as a full time Research Fellow in the University Department of Medicine and Therapeutics, Western Infirmary, Glasgow between August 1991 and July 1993. During this period, I was responsible for the organisation and day to day running of the studies contained herein as well as all aspects of data collection and the analysis of results. The research is entirely my own, although the work dealing with development of a gastrin/CCK-B receptor assay is part of an ongoing project which has subsequently been carried on by a further research student. The contribution of others, where appropriate, has been acknowledged overleaf.

All references have been personally consulted, with the exception of a few (largely historical) for which copies were not available or could not be traced.

DEFINITIONS

AOM	azoxymethane
B_{max}	maximal binding capacity
CCK	cholecystokinin
CEA	carcinoembryonic antigen
CYP	cytochrome P450
°C	degree(s) centigrade
DMH	1,2-dimethylhyrazine
DNA	deoxyribonucleic acid
f	femto
g	gram
G-17, G-34	gastrin-17, gastrin-34
GR	gastrin receptors
HP	*Helicobacter Pylori*
IC_{50}	50% inhibitory concentration
IQ	interquartile
K_d	dissociation constant
kDa	kilodalton
kg	kilogram
LI	labelling index
m	micro
m	milli
M	Molar
MBq	megaBecquerel
ml	millilitre
mol	mole
n	nano
ODC	ornithine decarboxylase
p	pico
PCR	polymerase chain reaction
PG	pentagastrin
PI	phosphotidylinositol
PKC	protein kinase C
RNA	ribonucleic acid
SD	standard deviation

PART ONE

INTRODUCTION AND LITERATURE REVIEW

CHAPTER 1

INTRODUCTION

1.1 COLORECTAL CARCINOMA

Colorectal carcinoma continues to be a major health problem in the industrialised world and is the second commonest cause of death from malignancy, accounting for fifteen percent of all cancers. In 1990, approximately 19,500 people in the United Kingdom alone died as a result of large bowel cancer (WHO Statistics Annual 1991). Indeed, almost one in twenty of the British population will develop colorectal cancer. Similarly, in the United States the most recent figures available (Boring *et al* 1992) estimate an annual incidence of approximately 156,000 new cases with 58,000 deaths annually. The incidence rises with age and, considering the proportional increase in the elderly population, it is likely that this disease will become even more common in years to come.

In the past three decades, overall mortality rates have improved only slightly, if at all (Stower and Hardcastle 1985), although the constancy of total deaths is possibly due in part to the increased population of elderly people with their higher incidence and case-fatality rate. Although advances in surgical management have reduced morbidity, the overall 5-year survival rate remains low at 35-40%, reflecting the fact that in the majority of cases the disease presents late in its natural history and is often beyond the limits of surgical resection at the time of diagnosis. It is therefore surgically incurable, the main determinant of prognosis being the extent of disease spread at diagnosis (Stower and Hardcastle 1985).

Improving the outlook of this condition is likely to depend on progress in several areas. Firstly, impressive recent advances in understanding the molecular genetic events underlying colorectal carcinogenesis have highlighted the importance of abnormalities in the expression of oncogenes and tumour suppressor genes in the carcinogenic process (Scott and Quirke 1993). Future research into these genetic changes may hopefully have potential applications in screening, predicting prognosis in individual cases and, ultimately, in gene therapy. For the time being, however, while the study of molecular genetic alterations in large bowel cancer has provided a wealth of

knowledge in our understanding of carcinogenesis in the colon it has not yet had any impact on the diagnosis or management of patients with sporadic colorectal neoplasia.

Secondly, because prognosis is related to extent of disease at diagnosis, efforts to diagnose large bowel cancer at an earlier stage are being made. Numerous studies have stressed the potential benefits of screening programmes for secondary prevention (Atkin *et al* 1993; Selby *et al* 1992) but screening (for example, by faecal occult blood testing and/or colonoscopy) remains a controversial issue and in the United Kingdom there is as yet no clear policy on screening for individuals at "average" risk, who constitute the vast majority of patients.

Alternatively, outlook could be improved by the development of improved or novel methods of treatment, especially therapies effective against locally advanced or systemic disease which cannot be cured by current surgical techniques or radiotherapy. The need for such treatment modalities has been emphasised by August *et al* (1984) who estimated that over fifty percent of deaths are due to disease which is beyond cure by primary surgery; the remaining deaths are due to recurrence of disease which was initially thought curable by surgery, but which is now beyond the limits of local therapy. While trials of adjuvant chemotherapy have demonstrated improvements in recurrence-free survival in patients with Dukes' C tumours (Saltz 1991), the benefits have been modest and at present chemotherapy seems likely to be restricted to this subgroup of patients. Furthermore, other systemic treatments such as biological response modifiers and immunotherapy have not fulfilled early promise and are not likely to be of practical value in the foreseeable future.

Given the limitations of current treatments for colorectal cancer and the lack of a safe, effective and widely applicable systemic therapy, an increasing volume of research in the last fifteen years has focused on the possible role of endocrine therapy for patients with large bowel and other gastrointestinal cancers. The concept that endocrine manipulation may be of therapeutic value in the treatment of a variety of malignancies is not new. Following the pioneering work of Beatson last century (Beatson 1896), who demonstrated the value of oophorectomy in inoperable breast cancer, the role of oestrogens in breast cancer has become established, as has the place

of the anti-oestrogen tamoxifen. Similarly prostatic carcinoma patients often benefit from treatment with anti-androgens as originally shown by Huggins *et al* (1941).

An appreciation of the endocrine significance of the gastrointestinal tract only developed with the isolation and purification of gastrin, cholecystokinin and secretin in the 1960's. Subsequently, a large and increasing number of peptides have been localised to cells of the digestive tract and shown to play regulatory roles in gastrointestinal function. The significance of many of these recently identified peptides has become clearer as a result of greater understanding of paracrine, neurocrine and autocrine modes of cell signalling in addition to the traditional endocrine concept of hormone action.

A number of gastrointestinal peptides, growth factors and non-gastrointestinal hormones are capable of affecting the proliferation and differentiation of epithelial cells in various parts of the digestive tract (Johnson 1987; Lemoine *et al* 1992). Many can also modify the growth of colorectal cancer cells, either *in vitro* or *in vivo* (Tahara 1990; Townsend *et al* 1987), raising the possibility of endocrine manipulation as a novel adjuvant treatment in colorectal carcinoma.

The antral hormone gastrin was the first for which trophic properties in the gastrointestinal tract were described. Subsequently, the majority of research into unravelling the role of hormones in the control of colonic epithelial proliferation, and the potential for endocrine manipulation of tumours arising from the colon (and other parts of the digestive tract), has concerned gastrin.

The remainder of this introduction will describe firstly the evidence for gastrin being an important physiological regulator of growth in the colon. Secondly, studies concerning the mitogenic effects of gastrin on colorectal cancer cell growth will be presented, along with discussion of the mechanisms of such effects. Thirdly, the state of our current knowledge regarding putative gastrin receptors will be reviewed along with the effects of newly developed specific gastrin receptor antagonists on the growth of colorectal cancer cells *in vitro* and *in vivo*. Studies of gastrin in human colorectal neoplasia will be described and then the aims of the current thesis presented.

CHAPTER 2

THE TROPHIC PROPERTIES OF GASTRIN

2.1 INTRODUCTION

Before considering the effects of gastrin itself, several general points regarding the regulation of gastrointestinal growth deserve mention. Firstly, the epithelium of the digestive tract is complex in that it is continuously and rapidly renewing itself. It exists in a delicate steady state where cell division is carefully balanced by exfoliation of cells into the lumen of the gastrointestinal tract as cells migrate and differentiate along the crypt.

Furthermore, division of cells in the gastrointestinal mucosa is restricted to distinct and definable regions of the epithelium, namely the crypt base in the colon. Division of undifferentiated crypt stem cells (the "vacuolated crypt base stem cell" in the colon) is followed by migration and differentiation of non-dividing cells as they pass up the length of the crypt, a process thought to take three to four days in the colon (Lipkin 1987). The rapid turnover of the epithelium, along with the distinct areas of mitosis make the digestive tract mucosa a useful experimental model for the study of epithelial growth control in general.

The control of growth in the gastrointestinal tract is, however, multifactorial and more complex than that in many other tissues. As well as being regulated by gastrointestinal peptides, the epithelium is also influenced by non-gastrointestinal hormones such as thyroxine, growth hormone and insulin (Johnson 1987). The mucosa is also constantly being exposed to ingested food, with its numerous effects. In addition to stimulating the release of various regulatory hormones and peptides, the very presence of food in the lumen of the gastrointestinal tract may have a trophic effect related to non-absorbed nutrients (luminal nutrition) and/or intestinal workload. A third mechanism postulated to play a role in the process is a local negative feedback loop from the crypt surface to the crypt base, of which little is known. Finally, neural control of colonic proliferation has been suggested (Tutton and Barkla 1982), but although this aspect of growth control has been studied in other tissues, relatively little research has been conducted in the gastrointestinal tract. Thus, a variety of diverse and interacting mechanisms are likely to operate in the normal regulation of colonic epithelial

proliferation and this has to be borne in mind when studying the influence of one particular aspect of the process in isolation. The results of such studies require careful interpretation before concluding that a given stimulus is directly responsible for a given effect because several other mechanisms may also be operative.

It is also important to consider the wide range of methods used to study growth in the digestive tract and the end-points and parameters used to define growth. The methods used vary greatly and include crude and insensitive measures such as estimates of tissue weight or volume, villous height and crypt depth, to simple and potentially inaccurate counts of mitotic figures. Many early studies of the trophic effects of gastrin used these methods. Later studies in the 1970's and 1980's utilised total DNA, RNA and protein contents to indicate a trophic response and many also measured tritiated thymidine uptake by tissues as a marker of DNA synthesis. This latter approach, although extensively adopted and still frequently used, has numerous pitfalls and may not accurately reflect DNA synthesis (reviewed in Maurer 1981). Studies based on this technique therefore have to be interpreted with caution. Furthermore, measuring total DNA or RNA content *in vivo*, or content of any particular substance for that matter, usually necessitates whole tissue measurements or, at best, analysis of mucosal homogenates. Even the gastrointestinal mucosa has a heterogeneous cell composition including significant numbers of non-epithelial cells and this may lead to erroneous results if measurements based on tissue homogenates are used. More accurate and sensitive methods of analysing cell proliferation in the gastrointestinal tract have been developed (Quinn and Wright 1990) and include bromodeoxyuridine labelling, immunocytochemical analysis of Ki67 antigen expression, quantification of crypt cell production rates in dissected but intact crypts and flow cytometric analysis of cell-cycle distributions.

Biochemical and molecular events associated with cell proliferation are increasingly being used as end-points in the study of trophic stimuli, for example ornithine decarboxylase activity and polyamine synthesis, membrane inositol phospholipid turnover and intracellular calcium measurements. All of these techniques have advantages and disadvantages in the study of cell turnover and the use of these

diverse methods of quantifying proliferation makes comparison of results from different studies difficult. The wide range of parameters for quantifying growth used by different research groups is probably responsible in large part for the failure to date to answer conclusively the question of whether gastrin is a trophic factor of physiological relevance to the colon.

Diverse experimental approaches and models have been employed in the study of gastrin's trophic role and this also compounds the problem of comparing the results of different investigators and drawing general conclusions about the hormone's importance. Most *in vivo* studies have been carried out in rats although mice (Balas *et al* 1985), guinea pigs (Håkanson *et al* 1986, 1988), hamsters (Chu *et al* 1992) and dogs (Willems *et al* 1972) have also been used. Even within the same species there is evidence that gastrointestinal tissues of young and adult rats behave differently in their response to gastrin (Poston *et al* 1991) and thus, the age and maturity of animals under study has to be considered when analysing experimental results.

Much experimental work in endocrinology has traditionally involved studying the effects of acutely or chronically elevating or lowering the level of the regulatory hormone of interest and observing the subsequent effects on tissue structure and function. Early studies of gastrin involved exogenous administration of gastrin or its synthetic analogue, pentagastrin, by injection (Johnson and Guthrie 1976; Johnson 1977; Mak and Chang 1976), or by continuous infusion using osmotic minipumps (Johnson *et al* 1975a; Ryberg, 1990). It has been argued that it is necessary to show that the endogenous hormone under study can produce the same effects in order to be of physiological relevance. Thus, a widely used alternative approach in endocrine research has been to remove surgically the source of a hormone, observe the subsequent effects and then try to reverse these effects by re-supplying the hormone exogenously. While many gastrointestinal peptides are expressed widely throughout the digestive tract the vast majority of circulating gastrin emanates from the gastric antrum, an anatomically well-defined area in rodents. As a result, many authors have used antrectomy to produce chronically low gastrin levels (Oscarson *et al* 1979; Dembinski and Johnson 1979; Chu *et al* 1992;). In contrast, a range of surgical procedures have

been successfully utilised to produce variable hypergastrinaemia in experimental animals. These include: vagotomy (Oscarson *et al*, 1979), fundectomy i.e. removal of the acid-secreting oxyntic mucosa (Chu *et al* 1992; Deveney *et al* 1983; Oscarson *et al* 1982) and antral exclusion (Oscarson *et al* 1979; McGregor *et al* 1983; Karlin *et al* 1985). Although trophic changes observed after such surgical manoeuvres have often correlated with parallel changes in gastrin levels, this does not prove a causal relationship and major surgical operations such as those mentioned invariably affect many aspects of gastrointestinal function, including subsequent food intake and weight gain, release of other regulatory peptides, acid-secretory capacity and the composition of intestinal flora. All of these factors may influence cell proliferation in the colon, either independently or in combination. Interpretation of such studies is therefore not always straightforward.

Non-surgical methods have been used to produce both elevated and lowered gastrin concentrations. Keeping rats fasted, but maintaining calorie intake and nitrogen balance with parenteral nutrition, results in significantly reduced gastrin (Johnson *et al* 1975b; Ryan *et al* 1979) and this model has proved useful for studying the effects of hormones without the presence of food in the gastrointestinal lumen. Alternatively, pharmacological blockade of the effects of gastrin on target tissues using specific gastrin receptor antagonists provides a convenient, non-surgical means of lowering 'effective' gastrin concentrations. In recent years an increasing number of specific and potent gastrin/CCK receptor antagonists have been developed and found applications in studying the trophic properties of gastrin on the mucosa of the gastrointestinal tract (Johnson and Guthrie 1984; Presti and Gardner 1993; Watson *et al* 1992).

Marked hypergastrinaemia can also be produced by dosing animals with acid-suppressing drugs, such as histamine-receptor antagonists or omeprazole. The powerful acid suppression so produced results in considerable and often chronic elevations in circulating gastrin (Håkanson *et al* 1986; Jansen *et al* 1990).

In summary, a large number of factors contribute to the lack of definitive conclusions about the role of gastrin as a physiologically important trophic hormone for the colon and other parts of the gastrointestinal tract. The complex control of

gastrointestinal epithelial proliferation and interspecies variation, along with differences in both study design and the choice of endpoints used to define and quantify growth have to be borne in mind when interpreting the data available to date.

2.2 EVIDENCE FOR THE TROPHIC PROPERTIES OF GASTRIN

2.2.1 Synthesis and Processing of Gastrin

Gastrin, the first gastrointestinal hormone to be discovered, was originally described by Edkins (1905) as a gastric acid-stimulating factor present in extracts of antral mucosa. It was nearly sixty years, however, before gastrin was purified as a hepadecapeptide by Gregory and Tracy (Gregory and Tracy 1964). Although it is now known that small amounts of gastrin may be synthesised by a wide variety of cells in diverse tissues (discussed more fully in Chapter 7), the vast majority of circulating gastrin originates from specialised G cells in the mucosa of the antrum and proximal duodenum. In recent years the complex biosynthetic pathway of gastrin has become well documented (Rehfeld and Hilsted 1992).

Gastrin is initially synthesised as preprogastrin, 101 amino acids in length. From this the signal peptide is enzymatically cleaved to yield progastrin which is then subjected to an orderly sequence of proteolytic cleavages (see Figure 2.1). The resulting caboxyl-terminal glycine-extended intermediate is then carboxyamidated to give the biologically active hormone (reviewed in Rehfeld and Hilsted 1992; Walsh 1993). Antral G cells thus release a number of different gastrins, although ninety percent is gastrin-17, five percent is gastrin-34 and smaller fragments of uncertain biological significance constitute the remainder. The crucial step for biological activity is alpha-amidation and the common C-terminal tetrapeptide amide (-Trp-Met-Asp-Phe-NH_2) has traditionally been regarded as responsible for all of the biological effects of gastrin. In 1994, however, several groups presented preliminary evidence that progastrin-derived glycine-extended intermediates may possess both trophic (Seva *et al* 1994a; Nègre *et al* 1994) and acid regulatory properties (Kaise *et al* 1994). This new discovery

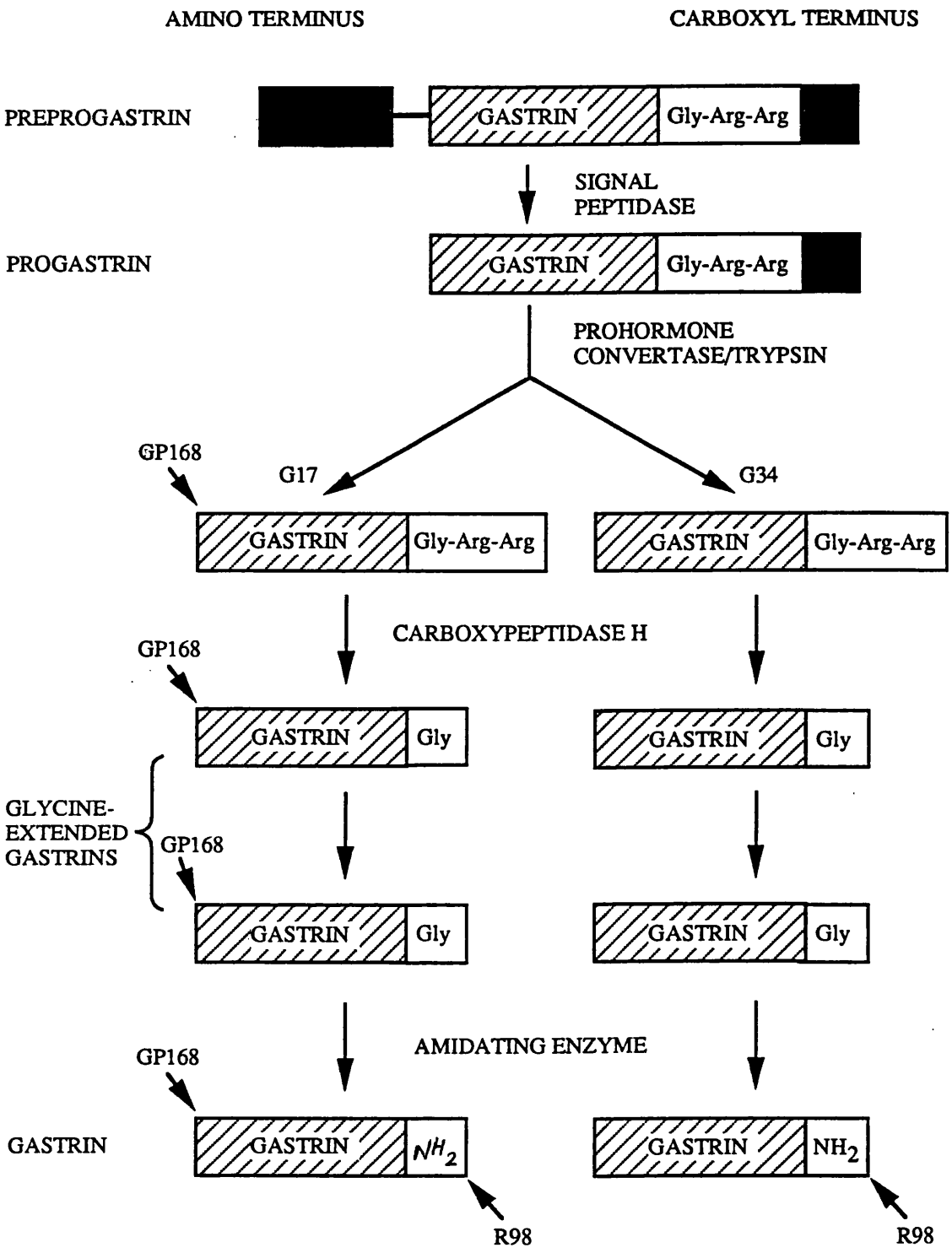


Figure 2.1

Schematic representation of gastrin processing. Arrows indicate binding sites of antigastrin antisera (R98, GP168) used in this study (see chapter 11).

is clearly going to develop further in the near future and is discussed later (Chapter 11). While this tetrapeptide sequence is also present in other members of the gastrin peptide family (most notably cholecystokinin), gastrin specificity is conferred by a tyrosine residue at position six.

2.2.2 Regulation of Gastrin Release

Just as complex as the synthesis of gastrin is the regulation of its release, with contributions from luminal, humoral and neural factors (Walsh 1994; Schubert 1993). Ingestion of a meal, especially one containing beef protein, is the most important stimulant of gastrin release. Peptone and amino acids derived from partial digestion of protein are particularly potent (McArthur *et al* 1988) whereas fat and carbohydrates are ineffective. Similarly, gastrin is released when intragastric pH rises above three and conversely antral acidification after a meal potently inhibits gastrin release. Vagal innervation stimulates gastrin release probably via release of gastrin-releasing peptide (GRP) from enteric nerve endings. Vagal reflexes can also inhibit gastrin release, although the mechanism of this is unclear. A number of gastrointestinal peptides also inhibit gastrin release, by far the most important of which is somatostatin, derived from antral D cells and which acts in a paracrine manner on adjacent G cells (Chiba and Kinoshita 1993). Although secretin, glucagon, vasoactive intestinal polypeptide and cholecystokinin can also inhibit gastrin release, their effects may be indirect via stimulation of somatostatin release and their physiological importance is uncertain.

2.2.3 Trophic Effects of Gastrin on the Stomach

(i) Historical

While the central importance of gastrin in gastric acid stimulation has long been acknowledged its putative role as a physiological trophic hormone for parts of the gastrointestinal tract mucosa has only become appreciated in the last twenty years or so. Initial clues to the mitogenic properties of gastrin came from clinical observations of the gastric mucosa in disease states which alter endogenous gastrin levels. Patients with

Zollinger-Ellison syndrome resulting from a gastrin-secreting endocrine tumour of the digestive tract have long been known to have thickened gastric rugae, gastric mucosal hyperplasia and an increased parietal cell mass (Ellison and Wilson 1967). Conversely, gastric biopsies in patients with a previous antrectomy show mucosal atrophy (Lees and Grandjean 1968) and these findings stimulated laboratory research into the growth properties of gastrin.

(ii) Experimental evidence

The first experimental work supporting the above findings came simultaneously from Crean *et al* in Edinburgh and Johnson *et al* in Texas in 1969. While studying the effects of pentagastrin injections on the gastric mucosa of rats Crean *et al* (1969) observed increased oxyntic mucosal height, weight and volume along with an increased parietal cell mass following treatment with the hormone. At the same time exogenous doses of pentagastrin which were submaximal for acid secretion were found to stimulate gastric mucosal protein synthesis as measured by [^{14}C]leucine incorporation into protein (Johnson *et al* 1969). Furthermore, this was independent of acid secretion as histamine was without effect and was also tissue-specific as no effect on either liver or skeletal muscle was observed.

Since these initial studies evidence for a physiologically important trophic role for gastrin in the stomach has steadily accumulated. Using *in vitro* tritiated thymidine uptake, pentagastrin was shown to stimulate DNA synthesis in the rat stomach by 275% (Johnson and Guthrie 1974), with significant uptake occurring after eight hours and reaching a maximum at sixteen hours. Because pentagastrin is a synthetic gastrin analogue which may also interact with cholecystokinin receptors, the same authors repeated their studies, showing that both G-17 and G-34 were of similar efficacy as pentagastrin. Both peptides doubled oxyntic mucosal DNA synthesis and were considerably more potent than pentagastrin on a molar basis. The presence or absence of sulphation appeared to make no difference to the effect (Johnson and Guthrie 1976; Johnson 1977). To counter criticisms that the doses of pentagastrin used were pharmacological (i.e. supranormal), they showed that doses less than half-maximal

(D_{50}) for gastric acid secretion were also trophic (Johnson *et al* 1975). Rats deprived of any oral dietary intake, but parenterally fed to maintain positive nitrogen balance, growth and weight gain, develop reduced mucosal weight and height in the oxyntic area of the stomach and elsewhere in the gastrointestinal tract (Johnson *et al* 1975). Such atrophy occurs in association with greatly reduced serum gastrin and antral gastrin content, the latter being thirty-fold lower compared to control animals (Johnson *et al* 1975). Of more relevance, coadministration of pentagastrin prevented these structural changes providing further evidence for a physiological trophic role for gastrin. From these experiments it seems that the presence of food in the gut lumen is necessary for the maintenance of both mucosal structure and antral gastrin stores. Similar effects of fasting on oxyntic gland weight, protein and DNA content and *in vivo* [^{3}H]thymidine incorporation, in association with a 90% decrease in serum gastrin, have been shown (Solomon, 1986). Again even the lowest dose of pentagastrin studied (which stimulated peak gastric acid output) was able to reverse some of these changes.

In other studies the physiological relevance of gastrin for the oxyntic mucosa has been examined by observing the effects of antrectomy on the trophic state of the mucosa. In the study of Dembinski and Johnson (1979), antrectomy lowered serum gastrin concentrations to around one third of control values. Similarly oxyntic gland DNA and RNA contents were reduced by 45% and 35%, respectively. Pentagastrin, at a dose of 250$\mu g.kg^{-1}$ four times daily, completely prevented such changes. Others have also found antrectomy to result in reduced gastric mucosal weight and height (Oscarson *et al* 1979) and content of nucleic acids (Dembinski *et al* 1987). As measured by *in vivo* [^{3}H]thymidine uptake, the labelling index in mice was increased after twelve days of G-17 injections (300$\mu g.kg^{-1}$), as were the number and diameter of parietal cells (Balas *et al* 1985). Interestingly cholecystokinin had no such effects but, in contrast to gastrin, it increased both the number of gastric chief cells and their total pepsin content. The effects of gastrin on the oxyntic mucosa occur not only in response to short-term studies involving intermittent injections of the hormone but are also seen following continuous infusions (Lehy *et al* 1979; Ryberg *et al* 1990). In the study of Ryberg *et al*, female Sprague-Dawley rats received a subcutaneous infusion of human G-17

continuously for twenty-eight days. Circulating gastrin levels were raised by a mean of 130% (range 50-285%), resulting in a 25% increase in oxyntic mucosal height relative to control animals.

(iii) Kinetics of the trophic response to gastrin

The time course of these trophic effects of gastrin has also been examined. Following a single intraperitoneal injection of pentagastrin ($250\mu g.kg^{-1}$), significant increases in rat oxyntic mucosal messenger RNA content were seen within one to two hours and remained elevated for up to twelve hours. Protein synthesis, as detected by [^{14}C]labelled amino acid uptake began within four hours, became significant by six hours (> 300% of control) and remained elevated for twelve to eighteen hours (Enochs and Johnson 1977). The same authors had previously shown maximal DNA synthesis to occur around sixteen hours after injection of pentagastrin (Johnson and Guthrie 1974). Others observed increases in mouse gastric labelling index at four hours and small intestinal increases at eight hours after gastrin treatment (Balas *et al* 1985).

(iv) Gastrin and enterochromaffinlike (ECL) cells

Gastrin exerts trophic effects not only on parietal cells but also has important effects on enterochromaffinlike (ECL) cells, the predominant endocrine cell in the oxyntic mucosa. These cells produce and store histamine along with other as yet unidentified peptides. Sustained hypergastrinaemia induced either by continuous infusions of gastrin (Ryberg *et al* 1990) or by chronic treatment with inhibitors of acid secretion such as ranitidine (Havu *et al* 1990) or omeprazole (Havu 1986; Ekman *et al* 1985), results in ECL cell hyperplasia with continued treatment. ECL carcinoid tumours develop in the stomachs of treated animals, although these changes can be prevented by prior antrectomy (Larsson *et al*, 1988). ECL cell hyperplasia and carcinoid tumour formation are also seen in patients with chronic hypergastrinaemia secondary to atrophic gastritis and/or pernicious anaemia (reviewed in Freston 1992). The role of gastrin in stimulating proliferation of ECL cells has been reviewed recently (Håkanson and Sundler 1991; Berlin 1991).

The notable effects of gastrin on ECL cells came to light during the development and testing of potent antisecretagogues such as ranitidine and omeprazole, widely prescribed drugs used in the management of peptic ulcer disease and other acid-related disorders. The endogenous hypergastrinaemia consequent upon administration of these drugs to experimental animals not only stimulates ECL cell proliferation but is also trophic to the oxyntic mucosa in general. Chronic omeprazole treatment has been shown to increase the numbers of parietal, chief and mucous cells in rat gastric glands by 25-30% (Håkanson *et al* 1986) while increases in oxyntic mucosal height and thickness are seen to occur in rats (Sundler *et al* 1986) and other species including chickens, hamsters and guinea-pigs (Håkanson *et al* 1988).There have, however, been fewer studies in humans with acid-suppressing drugs.

Changes in ECL cells after omeprazole therapy have been reviewed recently (Freston 1992) and no consistent pattern has been observed. There appears to be marked inter-individual variation in the ECL cell response to these drugs with no significant increase in the majority of patients. Little if anything is known about the trophic effects of acid-suppressing agents on the remainder of the oxyntic mucosa. The widespread use of these drugs, with around 1.7 million prescriptions per year in Scotland alone (Dr. P. Rutledge, Medical Prescribing Adviser, Lothian Health Board - personal communication) and the documented experimental effects of gastrin on gastric mucosal growth, gives study of the trophic effects of gastrin direct clinical relevance.

(v) Effects on gastric antrum

While the vast majority of experimental studies support a physiologically important trophic effect of gastrin on the mucosa of the oxyntic area of the stomach, they also demonstrate a complete lack of effect on the antral mucosa. Johnson (1977) found that intraperitoneal injections of pentagastrin ($250\mu g.kg^{-1}$) had no effect on antral mucosal [^{3}H]thymidine incorporation, while at the same time stimulating oxyntic mucosal uptake by 150% compared to control rats. Others have corroborated these findings (Balas *et al* 1985) and in the study of Casteleyn *et al*, gastrin actually reduced the labelling index in rat antrum despite being stimulatory to the oxyntic mucosa (Casteleyn *et al* 1977).

Although Lehy *et al* (1979) found gastrin to be equally effective at stimulating [^{3}H] thymidine uptake in both glandular and antral portions of the rat stomach, the weight of evidence would suggest that the antrum is not regulated trophically by gastrin. As Johnson has pointed out (Johnson 1987), this is perhaps not surprising given the neuroendocrine function of the antrum. As a rule endocrine organs such as thyroid, adrenals and gonads, do not respond trophically to the hormones that they themselves produce but are under the control of hormones originating elsewhere.

(vi) Studies using gastrin/CCK-B receptor antagonists

In recent years an increasing number of receptor antagonists for gastrointestinal peptides have become available as powerful tools with which to study many aspects of gastrointestinal function. At least eight classes of antagonists for the gastrin/CCK-B receptor have been described (Presti and Gardner 1993) and have been used mainly to classify receptor subtypes and study second messenger systems. To date their use in studies of gastrointestinal growth has concentrated mostly on their effects on the growth of malignant cells and not on normal epithelium (Watson *et al* 1991a; 1992). One of the first of these antagonists to be described was proglumide ((±)-4-(benzoylamino)-5-(dipropylamino)-5-oxopentanoic acid), a derivative of glutaramic acid. Although it has a relatively low affinity for the gastrin/CCK-B receptor it has been shown to inhibit pentagastrin-stimulated increases in DNA, RNA and protein content in rat oxyntic mucosa as well as the rate of DNA synthesis (Johnson and Guthrie 1984). It is notable that proglumide on its own had no effects on these parameters. The results of similar studies using newer and more powerful antagonists are still awaited. The effects of gastrin/CCK-B receptor antagonists on the growth of gastrointestinal tumour cells are discussed more fully later (Chapter 5).

2.2.4 Trophic Effects of Gastrin on Small Intestine

Whether gastrin is a physiologically relevant trophic hormone for parts of the gastrointestinal tract outwith the stomach is unclear, although many studies have addressed this issue and much has been published, virtually all of it using rodents as experimental models.

In addition to its lack of trophic effects on antral mucosa, the hormone does not appear to influence the growth of oesophageal epithelium (Mak and Chang 1976; Johnson 1977). Opinion is divided about possible growth-stimulatory effects in the small intestine and much of the conflicting data results from the widely differing methodologies used by different groups.

Some have used pooled mucosal samples from the entire length of the small intestine (Johnson *et al* 1975; Solomon 1986), while others have looked separately at duodenum, jejunum and ileum (Fatemi *et al* 1984; Balas *et al* 1985; Håkanson *et al* 1986). As discussed above, a variety of morphological, biochemical and kinetic techniques have been used to quantify trophic responses and this is likely to contribute to the uncertainty. In many studies the epithelium of the proximal duodenum has responded to gastrin in a manner similar to the oxyntic mucosa (Lehy *et al* 1979; Balas *et al* 1985; Johnson and Guthrie 1976; Johnson 1977) although these findings have not been unanimous (Fatemi *et al* 1984; Håkanson *et al* 1986; Ryberg *et al* 1990). Similarly, observed effects of gastrin on jejunal and ileal mucosae have been variable. Early studies by Johnson and co-workers (Johnson and Guthrie 1974; Johnson *et al* 1981) and others (Lehy *et al* 1979; Balas *et al* 1985) demonstrated trophism in jejunum and ileum but others have been unable to substantiate such findings (Oscarson *et al* 1977; Håkanson *et al* 1986; Ryberg *et al* 1990). Again choice of animal models and methods of quantifying growth are likely to have contributed to the disparity. The regulation of small bowel growth has been reviewed elsewhere (Johnson 1987; Vanderhoof 1993) and it is evident that, while gastrin is likely to be the major peptide regulating gastric mucosal growth, it is only one of a number of peptides capable of

stimulating cell renewal in the small bowel. Indeed it may well be of only secondary importance at this site.

2.2.5 Effects on the Exocrine Pancreas

Exogenous gastrin-17 or pentagastrin, as well as endogenous hypergastrinaemia induced by surgical procedures, have been shown to be trophic to the exocrine pancreas in animal models of pancreatic growth. Antrectomy resulted in reduced pancreatic weight, DNA synthesis and contents of DNA and RNA in rats and these changes were completely prevented by concomitant injections of pentagastrin (Dembinski and Johnson 1979). In contrast resection of the acid-secreting oxyntic mucosa (fundectomy) with consequent hypergastrinaemia significantly increases pancreatic weight and DNA content, as well as the proportion of cells in the DNA synthetic phase (S-phase) of the cell cycle (Chu *et al* 1993). Others have observed increases in exocrine pancreatic labelling index (Balas *et al* 1985) and DNA content (Poston *et al* 1991) after injections of exogenous gastrin-17 or pentagastrin.

A number of other peptides, most notably cholecystokinin, are also trophic to the exocrine pancreas (Solomon 1990; Mangino *et al*, 1992). Current evidence suggests that the stimulatory effects of gastrin on pancreatic growth are probably minor and of secondary importance in comparison to those of cholecystokinin. Experimental models of pancreatic growth and neoplasia and the contributions played by gastrin and cholecystokinin have recently been reviewed (Watanapa and Williamson 1993).

2.2.6. Trophic Effects of Gastrin on the Colon

(i) Effects of fasting and re-feeding

Once trophic properties of gastrin on the gastric mucosa of animals had been described, investigators began to examine the remainder of the gastrointestinal epithelium for evidence of gastrin-responsiveness and it was not long before effects on the colon were observed. In 1976 Mak and Chang found that a single injection of pentagastrin ($250\mu g.kg^{-1}$) in rats led to a five-fold increase in the labelling index of colonic crypts as

measured by autoradiography after *in vivo* [^{3}H]thymidine-labelling (Mak and Chang 1976). The labelling index was increased at all levels in the lower two-thirds of the crypts, the authors concluding that the hormone affected mainly the zone of proliferation-differentiation in the mid-crypt but not the fully differentiated cells at the top of the crypt. Interestingly, the stimulatory effects of pentagastrin were seen in fasted rats but not in their freely-fed counterparts. Fasting reduced the labelling index greatly and pentagastrin returned it to levels seen in freely-fed rats, the authors suggesting that in freely-fed rats circulating gastrin levels were sufficient for the maintenance of cell renewal with no further responsiveness to exogenous pentagastrin.

As noted above, fasting results in reduced cell proliferation and mucosal atrophy in the stomach and elsewhere in the gastrointestinal tract, even if adequate parenteral nutrition is given (Johnson *et al* 1975; Morin *et al* 1980). Refeeding results in a return of the proliferative parameters to their original level and while the local effects of food entering the colon may play a part ("luminal nutrition"), Delvaux *et al* showed that the response is seen in segments of colon defunctioned by a diverting colostomy (Delvaux *et al* 1984). Thus, an important humoral component is probable and gastrin has been suggested as a likely candidate as antral and serum gastrin are dramatically reduced by fasting, the effects of which can be reversed by exogenously supplying the hormone (Mak and Chang 1976; Majumdar 1984). To test this the same group examined the effects of fasting and re-feeding on [^{3}H]thymidine uptake in the defunctioned colon of rats with or without prior antrectomy (Haentjens *et al* 1986). Antrectomy abolished the post-prandial rise in serum gastrin and yet re-feeding still resulted in increased DNA synthesis in the defunctioned colon, suggesting that gastrin is either not the humoral mediator or only one of several released postprandially and capable of stimulating colonic mucosal proliferation. In support of this, Goodlad *et al* found no significant correlation between plasma gastrin levels after re-feeding and crypt cell production rate in the colon (Goodlad *et al* 1983).

(ii) Effects of antrectomy

A number of studies have examined the effect of removing the endogenous source of gastrin by antrectomy. Dembinski *et al* subjected rats to either antrectomy or sham operation and treated half the antrectomised animals with pentagastrin ($250\mu g.kg^{-1}$ eight hourly) for one week after recovery from surgery (Dembinski and Johnson 1979). Compared to sham-operated controls antrectomy reduced serum gastrin by 62% and colonic weights in these animals were also approximately 15% lower. DNA synthesis (*in vitro* [^{3}H]thymidine uptake) in colonic mucosal scrapings was only 59% of that in controls and similar reductions in total RNA and DNA contents were also observed. In addition, the changes in all these parameters were completely prevented by pentagastrin treatment.

In a study using small numbers of hamsters (n=8 per group), Chu *et al* found that antrectomy lowered fasting plasma gastrin by 50% both five and twenty-five days after the procedure (Chu *et al* 1992). Using autoradiography and quantitative histological analysis of the colonic mucosa, colonic mucosal [^{3}H]thymidine uptake was significantly reduced at day five compared to sham-operated control animals, and the number and percentage of goblet cells per crypt were lower by day twenty-five.

Other authors have been unable to confirm these findings. In one study antrectomy (with proximal duodenectomy) did not result in a fall in colonic weight, DNA synthesis or content of nucleic acids (McGregor *et al* 1983). In this study, however, serum gastrin did not fall after antrectomy for reasons which the authors could not explain. It is impossible to negate a role for gastrin from these results. Likewise, Deveney *et al* (1983) found that antrectomy lowered post-prandial gastrin levels but had no effect on fasting gastrin. Again antrectomy affected neither colonic weight nor mucosal thickness at three months postoperatively, although the parameters measured are relatively crude and insensitive markers of proliferative status. Thus, definite conclusions about the effect of removing endogenous gastrin on the trophic state of the colon are difficult to draw because of methodological differences in these studies.

(iii) Effects of surgically-induced endogenous hypergastrinaemia

The effects of surgically-produced chronic endogenous hypergastrinaemia on the colon have also been assessed. A variety of surgical procedures have been used to induce hypergastrinaemia, all of them by interfering with acid-mediated inhibition of antral gastrin release.

In 1976 MacGregor and Way reported that antral implantation into the colon, which raised fasting gastrin five-fold and postprandial levels three-fold, resulted in a small but significant increase in colonic weight (McGregor and Way 1976). Furthermore, antral implantation with similar elevations of gastrin concentrations, increased transverse colonic weight by 27% and mucosal thickness after twelve weeks (Deveney *et al* 1983). These changes also occurred in rats with both antral implantation and total gastrectomy and were therefore independent of the presence of acid.

Antral exclusion, consisting of gastric partition across the antrum and gastrojejunostomy, diverts the gastric contents past the antrum and also causes chronic hypergastrinaemia. In this way serum gastrin was elevated four-fold compared to sham-operated control rats, in association with a two-fold increase in DNA and RNA content of colonic mucosa (McGregor *et al* 1983). Rates of synthesis of DNA (*in vivo* [^{3}H]thymidine uptake) and RNA (*in vivo* [^{14}C] uptake) were increased two and-a-half and four-fold, respectively).

Other authors have produced endogenous hypergastrinaemia by fundectomy with removal of the entire oxyntic gland area of the stomach. In an early study Oscarson *et al* (1979) used various surgical procedures, including fundectomy, to elevate endogenous gastrin levels. Despite mean gastrin levels being three and-a-half times those in unoperated controls, only minor non-significant increases in large bowel mucosal weight, DNA and RNA content were seen. Beauchamp *et al* reported in an abstract that fundectomy resulted in a 21% increase in total colonic weight after two to six weeks (Beauchamp *et al* 1985a). This effect was no longer apparent in rats followed for five to seven months and no significant increase in colonic DNA, RNA or protein content was observed at either time point. Unfortunately the *whole* colon was used for

analysis, including submucosa and muscle layers, so significant effects on the epithelium alone could have been missed for technical reasons. More recently Chu *et al* subjected hamsters to either antrectomy, fundectomy or sham operation. In addition to the effects of antrectomy discussed above (Chu *et al* 1992), fundectomy raised basal plasma gastrin four-fold compared to controls and doubled DNA synthesis by day five. By day twenty-five, rates of DNA synthesis were similar to control animals but there were significant increases in crypt size (30%), number of goblet cells per crypt (46%) and the percentage of goblet cells per crypt (13%). Although surgical manipulations such as those described above are likely to have a variety of effects and alter levels of other hormones and growth factors, it is likely that chronic endogenous hypergastrinaemia does indeed stimulate colonic hyperplasia in animals. Furthermore, the fact that these changes occur at gastrin levels similar to those seen postprandially argues in favour of the trophic response being physiological rather than a pharmacological phenomenon.

(iv) Effects of exogenous gastrin

Shortly after Mak and Chang reported the trophic effect of pentagastrin on the colon (Mak and Chang 1976), Johnson compared different doses of pentagastrin and found that DNA synthesis (*in vitro* [^{3}H]thymidine uptake) in colonic mucosal scrapings was significantly stimulated even at the lowest dose tested (62.5μg.kg^{-1}), a dose which is submaximal for gastric acid secretion (Johnson 1977). Maximum label incorporation (150% compared to control) occurred at 250μg.kg^{-1} and increasing the dose further was significantly less effective. The author also noted that for each dose greater stimulation occurred in the colon than in the duodenum. Intriguingly, the same group have also shown that a continuous infusion of gastrin-17 into the ileal lumen resulted in a doubling of colonic DNA synthetic rate even though no rise in circulating gastrin was seen (Johnson *et al* 1981).

Fatemi *et al* (1984) compared the effects of intermittent injections of either pentagastrin or a single injection of porcine gastrin on colonic mucosal proliferation. In this detailed study two different strains of rats (Lewis and Wistar) were compared, a range of different doses were used and triplicate samples of fifty individually dissected

crypts were analysed for DNA synthesis ([^{3}H]thymidine uptake *in vivo*) and protein synthesis ([^{14}C]leucine incorporation *in vivo*). While pentagastrin at supraphysiological doses (1000μg.kg^{-1}) was trophic, gastrin was trophic at all doses (10-40μg.kg^{-1}) tested. Regression analysis suggested a significant quadrantic relationship between gastrin dose and the number of crypts per colon.

These growth-stimulating effects of gastrin on the colon have, in one study, been prevented by the relatively weak gastrin/CCK-B receptor antagonist proglumide (Johnson and Guthrie 1984). While pentagastrin injections doubled the rate of mucosal DNA synthesis, proglumide completely abolished this response in the same dose range as that required to inhibit the binding of gastrin to its receptor. Similar studies using newer antagonists with greater specificity and higher affinity for the gastrin/CCK-B receptor are awaited and will hopefully shed further light on the trophic role of gastrin on the colon.

Like the studies described above using surgical models of altered gastrin levels, experimental work using exogenous gastrin have not produced unanimous results. Solomon (1986) injected rats with a range of doses of pentagastrin and measured gastric acid output to establish the doses which stimulated maximal acid output. Using doses maximal and supramaximal for acid secretion, no effect on colonic weight, content of protein or DNA, nor [^{3}H]thymidine uptake was seen. More recently Ryberg *et al* studied the trophic effects of continuous intravenous gastrin infusions for twenty-eight days on proliferation on all parts of the digestive mucosa (Ryberg *et al* 1990). While two to four-fold mean rises in circulating gastrin levels were achieved, no increases in either colonic mucosal wet weight or thickness were observed. Unfortunately, more sensitive and subtle indices of trophism, such as DNA synthesis, labelling index or crypt cell production rate, were not studied. In a recent abstract with few details, neither gastrin nor pentagastrin by continuous infusion for one week was found to be trophic to rat colon even though effects on gastric fundus were observed (Ekundayo *et al* 1993).

(v) Effects of drug-induced endogenous hypergastrinaemia

The effects of omeprazole-induced hypergastrinaemia on colonic mucosal growth have also been examined. In 1991 Pawlikowski *et al* (1991) treated rats with omeprazole with an approximate two-fold rise in gastrin levels. Compared to control rats the colonic mitotic index (vincristine-arrested metaphases) in omeprazole-treated rats was doubled. Also, the weak gastrin receptor antagonist proglumide prevented the effects of omeprazole. It should be noted that proglumide on its own seemed to stimulate mitotic activity, possibly because it possesses weak intrinsic agonist activity in addition to its antagonist properties. In another study, thirty days of omeprazole treatment elevated gastrin levels twelve-fold, with an associated increase in colonic polyamine content (74%) and a small but statistically significant increase (7%) in crypt cell production rate (Gray *et al* 1993). In contrast, Håkanson *et al* were unable to demonstrate any effect of omeprazole-induced hypergastrinaemia on colonic wet weight or mucosal thickness in either rats (Håkanson *et al* 1986), hamsters or chickens (Håkanson *et al* 1988).

Very recently, the effects of this widely used drug on human colonic proliferation have been examined. In a small study of eight patients taking omeprazole for treatment of Barrett's oesophagus, omeprazole increased both *in vitro* [^{3}H]thymidine uptake in colonic biopsies and ornithine decarboxylase activity, although the increases did not reach statistical significance (Murphy *et al* 1993). The authors concluded that omeprazole-induced hypergastrinaemia did not increase colonic mucosal proliferation, although there was considerable inter-individual variation in the rise in gastrin resulting from treatment. Patient numbers were also very small (thus, a type 2 error cannot be excluded) and the methodology used can be prone to inaccuracies.

Despite the considerable volume of reported work, no clear consensus regarding a physiological role for the hormone in the colon has been reached. Nevertheless, many investigators have examined the effects of gastrin on colorectal cancer cells. The following chapters review this work, both animal studies and *in vitro* cell culture studies.

CHAPTER 3

TROPHIC EFFECTS OF GASTRIN ON COLORECTAL CANCER CELLS *IN VITRO*

3.1 GASTRIN AND COLORECTAL CANCER CELLS *IN VITRO*

The use of *in vitro* cell culture systems provides a powerful method for studying in a carefully controlled environment the cellular, biochemical and genetic mechanisms involved in regulating cell growth. It is only relatively recently that techniques became available to grow normal gastrointestinal epithelial cells in pure populations without the problems of fibroblastic overgrowth and short survival of the cultured cells (Moyer 1983; Moyer *et al* 1990).

The earliest report was that of Aspegren *et al* in 1977 who studied *in vitro* [^{3}H]thymidine uptake into freshly disaggregated gastric carcinomas in response to pentagastrin. In this study (Aspegren *et al* 1977) they also examined two fresh human colonic carcinomas although few details are given regarding the tumour characteristics, assay methodology or whether the cells were passaged or not prior to use. While gastrin stimulated [^{3}H]thymidine uptake in three of five gastric cancers, no effect on the two colonic cancers was observed. A few years later Murakami and Masui (1980) managed to establish the growth of a human colon cancer (HC84S), normally transplanted serially in nude mice, in cell culture using a hormone-supplemented, serum-free medium. They then studied the trophic effects of a large number of diverse hormones and noted that gastrin at a concentration of 50pM stimulated growth of the cells compared to control cells. The response to gastrin (14% increase) was small relative to the effects of insulin (134%) and glucagon (57%) but nevertheless the hormone dependence of colonic cancer cells *in vitro* had been demonstrated. The first major study of the effects of gastrin on human colonic cancer cells *in vitro* was that of Sirinek *et al* (1985) who found that pentagastrin at a dose of 5μg.ml^{-1} stimulated a 29% increase in cell numbers compared to control cells using the established human colonic carcinoma cell line HT-29. This increase above and beyond that seen in untreated control cells over the same period (310%) was small, however, and did not reach statistical significance. In the same study they also looked at four newly established cultures from human colonic carcinomas along with five normal human colonic epithelial cell cultures. In contrast to the HT-29 cells these cultures were "early

passage" and pentagastrin ($5\mu g.ml^{-1}$) increased both normal and malignant cell numbers compared to controls by means of 65% and 59%, respectively. In support of this Watson *et al* (1989a) found that two freshly established human colorectal cancers responded trophically to $250\mu g.L^{-1}$ pentagastrin at passage two (with a two-fold increase in [^{75}Se]selenomethionine uptake). In contrast, none of seven established colorectal cell lines, including HT-29, exhibited a trophic response to pentagastrin in doses ranging from $1.5\text{-}1500\mu g.L^{-1}$. Suspension cultures of clumps of three freshly disaggregated human colonic carcinomas also showed a significant trophic response to pentagastrin compared to control cultures. The authors concluded that trophic responses to gastrin are lost during *in vitro* culture and that gastrin responsiveness may not be seen if only long-established cell lines are studied.

In the last ten to fifteen years a considerable number of studies have examined the trophic effects of gastrin on colorectal cancer cells *in vitro* with conflicting results and these are summarised at the end of this chapter in Table 3.1. Some authors have found gastrin responsiveness in some cell lines but not others while a given cell line has responded differently to gastrin in different studies. Many factors, including the duration of culture and number of passages of a given cell line, are likely to contribute to these conflicting results. In addition to methodological differences such as choice of culture media, incubation conditions, addition of varying supplements to media and parameters used to measure proliferation, several other important observations are worth highlighting. Firstly, several authors have demonstrated the importance of "synchronising" cells in culture prior to studying the trophic effects of gastrin and other exogenous factors (Kusyk *et al* 1986; Watson *et al* 1988, 1989a; Guo *et al* 1990). Synchronisation refers to the exposure of cells to excess thymidine prior to study, with the result that all cells are held at the G_1 phase of the cell cycle. Removal of the thymidine prior to addition of the trophic factor(s) under investigation releases the cells from growth-arrest. In one study asynchronous LoVo cells showed an inconsistent response to gastrin which varied from stimulation to actual *inhibition* of growth in different experiments (Kusyk *et al* 1986). When the cells were synchronised, however, reproducible and pronounced trophic effects were observed as measured by both

[^{3}H]thymidine uptake and counts of cell numbers. Furthermore, gastrin was effective even at a concentration as low as 1.8 x 10^{-10}M, similar to the upper level seen postprandially in humans and this dose increased cell numbers by 44% at forty-eight hours relative to control cells. Similarly Guo *et al* (1990) found that gastrin had no effect on either total cell number or [^{3}H]thymidine uptake by mouse colon cancer (MC-26) cells but was significantly trophic in doses ranging from 1-25 x 10^{-9}M when the cultures were synchronised. In contrast Watson *et al* (1988) found that neither LoVo nor ST16 cells responded to gastrin irrespective of whether they were synchronised or not. These were long-established cell lines and as the authors pointed out, gastrin responsiveness may have been lost for this reason. Thus, several studies have highlighted the importance of cell synchronisation in allowing the trophic effects of exogenous compounds such as gastrin to be revealed when they would otherwise not have been seen. Results of studies using asynchronous cells therefore have to be interpreted in this light.

A second important point is the effect of serum supplementation on the growth-stimulating properties of gastrin. In addition to highlighting the importance of synchronisation, Kusyk *et al* (1986) observed that cells were more responsive to gastrin in the presence of 10% foetal bovine serum (FBS), with maximal [^{3}H]thymidine uptake (220%) occurring at a dose of 7.2 x 10^{-10} M. While cells grown in the absence of serum had a similar maximal [^{3}H]thymidine uptake (200%), this required a gastrin dose of 3.6 x 10^{-9} M i.e. five-fold higher, leading the authors to suggest that serum either contained a factor which enhanced the action of gastrin or upregulated the gastrin receptors present on the cells. In another study neither gastrin nor 17β-oestradiol stimulated the growth of LoVo or HCT-15 human colorectal cancer cells initially when grown in ordinary 10% serum-supplemented medium (Kiss *et al* 1991). When these "hormone-insensitive" cells were repeatedly passaged in medium containing only 1% serum but also containing gastrin, responsiveness to further added exogenous gastrin was restored. In stark contrast to the findings of Kusyk *et al* noted above, Guo *et al* (1990) observed that synchronised mouse colon cancer (MC-26) cells showed a trophic response to gastrin only in the complete *absence* of foetal bovine serum. Thus, the

effects of serum on cellular responses to gastrin appear to be complex, possibly partly due to the complex composition of serum itself, the different animal sources used in its manufacture and the well-recognised variations noted in different batches of serum from the same source. That serum should modulate the effects of gastrin is not surprising given the possible requirements of hormones for receptor-binding and stimulation and the vast number of other hormones, peptides and co-factors present in serum. Colorectal cancer cells contain a diverse array of peptide and steroid hormone receptors (Frucht *et al* 1992; Alford *et al* 1979; Singh *et al* 1993b). Few studies have specifically addressed the possible synergistic or co-operative actions of other hormones on the trophic effects of gastrin but in one study gastrin and transforming growth factor-alpha (TGF-α) had additive growth stimulatory effects on one of three human colorectal carcinoma cell lines (Durrant *et al* 1991). Synergistic effects were also observed on a human gastric carcinoma cell line (MKN45) in the same study.

Finally it should be noted that, while many studies used synthetic human G-17 to stimulate growth of cells, a considerable number actually used pentagastrin (Aspegren *et al* 1977; Sirinek *et al* 1985; Tanaka *et al* 1986; Eggstein *et al* 1991). While the biological activity of gastrin is thought to reside in the C-terminal tetrapeptide, receptor affinity is influenced by the tertiary structure of the whole molecule. Indeed pentagastrin has a lower binding-affinity than G-17 for gastrin receptors (Takeuchi *et al* 1979) and is far less potent at stimulating DNA synthesis, at least in rat gastric mucosa (Takeuchi *et al* 1980). Also, Mauss *et al* (1994) found that G-17 at concentrations of 10^{-9}-10^{-12}M significantly increased HT-29 cell numbers whereas pentagastrin at concentrations ranging from 5×10^{-16} - 5×10^{-5}M had no effect. Thus, the results of some studies using pentagastrin alone could conceivably have been different if G-17 had been used. Gastrin has also been extensively studied *in vitro* as a potential proliferative hormone for gastric and pancreatic carcinomas (reviewed in Tahara 1990; Townsend Jr *et al* 1988; Morris *et al* 1989). It may also be a growth factor for small cell lung cancer cells (Sethi and Rozengurt 1992; Yoder and Moody *et al* 1987). Further discussion of the growth-stimulating effects of gastrin in these cancers is beyond the scope of this thesis.

In summary, a wealth of data dealing with the growth-stimulating effects of gastrin on colorectal cancer cells *in vitro* has accumulated in recent years. While overall the results of such studies suggest that gastrin is capable of acting as a trophic factor for some colorectal tumours under certain conditions, this is not universally the case. Also, the physiological relevance of these findings has not been convincingly demonstrated and at present it cannot be concluded from *in vitro* studies that gastrin is an important trophic factor of clinical relevance in colorectal cancer. The next chapter reviews evidence regarding the trophic effects of gastrin on colorectal tumours *in vivo* using animal models.

Cell line	Species	Hormone	Dose Range	Assay	+/-	Reference
HT29	human	PG	5-25mg.L^{-1}	cell counts	+/-	Sirinek *et al* 1985
HT29	human	G-17	0.4-4000 x 10^{-12}M	[^{3}H]thymidine uptake	+	Smith *et al* 1988
HT29	human	PG	1.5-1500µg.L^{-1}	[^{75}Se]selenomethionine uptake	-	Watson *et al* 1988
HT29	human	G-17	0.02-10µg.L^{-1}	[^{75}Se]selenomethionine uptake	+	Watson *et al* 1989a
HT29	human	PG	5mg.L^{-1}	cell counts	+	Moyer *et al* 1990
HT29	human	PG G-17	0.5 x 10^{-15} - 10^{-2}M 10^{-15}-10^{-7}M	cell counts	+ (G-17) - (PG)	Mauss *et al* 1994
HT29	human	G-17	10^{-12} - 10^{-6}M	cell counts	+	Ishizuka *et al* 1994
LoVo	human	G-17	0.72-28.8 x 10^{-9}M	cell counts, [^{3}H]thymidine uptake	+	Kusyk *et al* 1986
LoVo	human	PG G-17	1.5-1500µg.L^{-1} 0.2-10µg.L^{-1}	[^{75}Se]selenomethionine uptake	-	Watson *et al* 1988
LoVo	human	G-17	0.02-20mg.L^{-1}	[^{75}Se]selenomethionine uptake	-	Watson *et al* 1989a

Table 3.1. (3 pages). Published studies of the effects of gastrin on colorectal cancer cells in vitro.
PG = pentagastrin, G-17 = gastrin-17. + trophic effect, - no effect. *Actual inhibition of growth.

Cell line	Species	Hormone	Dose Range	Assay	+/-	Reference
LoVo	human	PG	$10mg.L^{-1}$	cell counts	-	Eggstein *et al* 1991
LoVo	human	G-17	10^{-9} - $10^{-7}M$	MTT assay	+	Kiss et al 1991
LoVo	human	G-17	10^{-12} - $10^{-6}M$	cell counts	+	Ishizuka *et al* 1994
LS174T	human	PG	$10mg.L^{-1}$	cell counts	-	Eggstein *et al* 1991
LS174T	human	PG	$1.5\text{-}1500\mu g.L^{-1}$	[^{75}Se]selenomethionine uptake	-	Watson *et al* 1988
HCT116	human	PG G-17	0.5×10^{-15} - $10^{-2}M$ 10^{-15}-$10^{-7}M$	cell counts	+ (G-17) - (PG)	Mauss *et al* 1994
HCT116	human	G-17	5×10^{-9} - $5 \times 10^{-8}M$	MTT assay	+	Blackmore *et al* 1994
HCT116	human	G-17	10^{-12} - $10^{-6}M$	cell counts	- *	Ishizuka *et al* 1994
T84	human	PG G-17	0.5×10^{-15} - $10^{-2}M$ 10^{-15}-$10^{-7}M$	cell counts	+ (G-17) - (PG)	Mauss *et al* 1994
HC84S (T84)	human	G-17	$5 \times 10^{-11}M$	protein content	+	Murakami *et al* 1980

Cell line	Species	Hormone	Dose Range	Assay	+/-	Reference
SW1116	human	PG	$10mg.L^{-1}$	cell counts	-	Eggstein *et al* 1991
SW403	human	PG	$10mg.L^{-1}$	cell counts	+	Eggstein *et al* 1991
HCT15	human	G-17	10^{-9} - 10^{-7}M	MTT assay	+	Kiss et al 1991
WiDr	human	G-17	10^{-10} - 10^{-9}M	[^{3}H]thymidine uptake	+	Smith *et al* 1993
DLD-1	human	G-17	10^{-7} - 10^{-6}M	not stated	+	Singh *et al* 1993a
COLO320	human	G-17	10^{-12} - 10^{-6}M	cell counts	+	Ishizuka *et al* 1994
C146	human	G-17	$3\mu g.L^{-1}$	[^{75}Se]selenomethionine uptake	+	Durrant *et al* 1991
C168,C170, HCT8,HRT18	human	PG	$1.5\text{-}1500\mu g.L^{-1}$	[^{75}Se]selenomethionine uptake	-	Watson *et al* 1988
MC-26	rat	G-17	0.5 - 25 x 10^{-9}M	MTT assay, [^{3}H]thymidine uptake	+	Guo *et al* 1990
not stated	rat	G-17	10^{-10} - 1.2 x 10^{-9}M	MTT assay	+	Yactayo *et al* 1991

Table 3.1 contd. (page 3 of 3).

CHAPTER 4

TROPHIC EFFECTS OF GASTRIN IN ANIMAL MODELS OF COLORECTAL NEOPLASIA

4.1 INTRODUCTION

The use of *in vitro* culture methods allows us to study pure populations of cancer cells in isolation and under rigidly controlled conditions. Such techniques inevitably have drawbacks and inherent limitations, being especially open to the criticism that cancer cells *in vitro* may be unrepresentative of their counterparts *in vivo* and may behave differently *in vitro*. Tumours *in vivo* also have heterogeneous cell compositions and interact with their host in many complex ways which cannot be studied (or controlled for) *in vitro*. These drawbacks of cell culture methods along with the practical problems and ethical constraints of human studies have resulted in much effort being expended in recent years into developing suitable animal models of colorectal cancer. Although hampered by the rarity of spontaneously occurring adenocarcinoma of the colon in animals several different experimental models of colorectal cancer now exist, each with its own strengths and weaknesses.

These models not only allow study of the numerous steps in the carcinogenic process, identification of novel tumour markers and understanding of the role of the immune system in tumourigenesis, but also allow investigators to manipulate factors such as diet and intestinal bacteria. The effects of novel therapies on tumour growth and survival can be assessed and, in this context, the use of gastrin-receptor antagonists will be discussed later (Chapter 6). Similar to *in vitro* methods, experimental conditions in animal models can be strictly controlled.

4.2 ANIMAL MODELS OF COLORECTAL NEOPLASIA

Virtually all experimental studies have used rodents and, unless otherwise stated, what follows refers to rodents. Traditionally the two main animal models employed have been chemical carcinogen-based systems and transplantable "xenograft" systems involving nude mice (Lamont and O'Gorman 1978; Galloway 1989; Pories *et al* 1993). Very recently investigators have begun to take advantage of advances in molecular biology to study genetic factors in colonic carcinogenesis, using chimeric animals, transgenic mice

and "reconstruction" models (Pories *et al* 1993). These latter models will not be discussed further.

4.2.1 Chemical Colorectal Carcinogenesis

At least five structurally different classes of compound can induce cancer in the large intestine and these have been reviewed elsewhere (Lamont and O'Gorman 1978; Weisburger and Fiala 1983; Rogers and Nauss 1985). N-methyl-N^1-nitro-N-nitrosoguanidine (MNNG) differs from the other carcinogens in that it acts directly on the colonic mucosa following intrarectal instillation and produces cancers without prior metabolic activation by the host animal. In spite of this, hydrazine derivatives such as 1,2-dimethylhydrazine (DMH) and azoxymethane (AOM) have been shown to be the most potent inducers of colonic tumours (which are also transplantable) and have become the most widely used for studies of chemical colorectal carcinogenesis. As this model has been used in the present thesis, it will be described further before the results of studies involving gastrin in experimental colorectal cancer are discussed.

(i) Hydrazine carcinogenesis

The story of the discovery of hydrazines as potent colonic carcinogens has been recounted in detail elsewhere (Lamont and O'Gorman 1978; Galloway 1989). Briefly, while investigating the unusually high incidence of amyotrophic lateral sclerosis on the island of Guam, Lacquer *et al* (1963) noted that cycad flour, from the nut of the plant *Cycas circinalis*, formed a major component of the local diet. When fed to rats, however, no neurological lesions were produced but a small number of animals developed colonic adenocarcinomas. Cycasin was shown to be the β-glucoside of the chemically unstable compound methylazoxymethanol. Separately, Druckrey had discovered that 1,2-dimethylhydrazine also produced a similar pattern of intestinal tumours and was structurally similar to cycasin (Druckrey 1970). Further work (reviewed in Lamont and O'Gorman 1970; Rogers and Nauss 1985) demonstrated the carcinogenicity of azoxymethane and the likely metabolic pathway from

dimethylhydrazine to the highly unstable methyl carbonium ion, which alkylates cellular macromolecules including DNA. The metabolic pathway of hydrazine carcinogens is illustrated in Figure 4.1. The potency, relative ease of synthesis and chemical stability of azoxymethane (AOM) has made it the most widely used of this group of carcinogens.

Exactly why 1,2-dimethylhydrazine and azoxymethane should exhibit their characteristic organotropism following parenteral administration remains to be fully explained. Initial suggestions that the carcinogen underwent biliary excretion as a glucuronide conjugate, to be split by intestinal bacteria with carcinogen release in the colonic lumen, have not been substantiated. Segments of colon from which the faecal stream has been surgically diverted still develop tumours in response to hydrazine carcinogens (Campbell *et al* 1975). Thus important factors may be either the vascular delivery of carcinogen to the colon or properties inherent in colonic epithelial cells such as cell-cycle kinetics or expression of particular xenobiotic metabolising enzymes.

In rats and mice both DMH and AOM are highly effective as colonic carcinogens and even a single dose is sufficient for tumour induction after a prolonged latent period. Tumourigenesis is dose-related in terms of both the number of tumours formed and their rapidity of onset. In most studies doses of 10-20mg.kg^{-1} body weight are given by weekly subcutaneous injection for approximately twelve weeks (Shamsuddin 1983). Doses in this range preferentially induce colonic lesions while higher doses result in a higher yield of extracolonic abnormalities including benign renal cysts, hepatic haemangioendotheliomas, adenocarcinomas of the duodenum and squamous carcinomas of the external ear.

Hydrazine derivatives have recently been shown to induce colon cancers in monkeys as well as rodents (Beniashvili *et al* 1992) but are not known to be relevant carcinogens for human colorectal cancer. Substituted hydrazines are used as components of rocket fuel, as a ripener for pineapples and are found in some edible mushrooms (Lamont and O'Gorman 1978). Although evidence for a role in human disease is lacking, there are important similarities between the colonic tumours induced by hydrazines and their human counterparts, both in morphology and in their propensity to develop in the distal colon. The majority of lesions are adenomas and non-mucinous

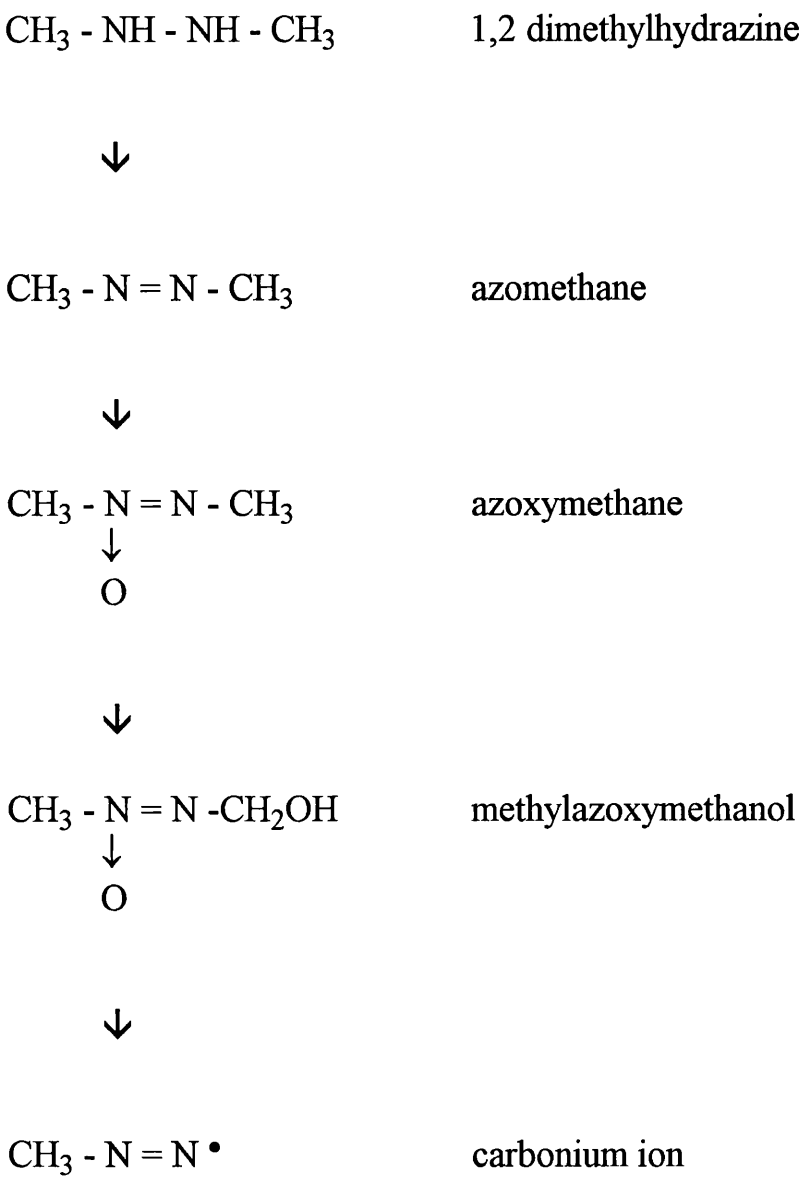


Figure 4.1. Metabolic pathway of hydrazine carcinogens.

adenocarcinomas, the latter being locally invasive. Less often aggressive mucinous carcinomas are also found (Shamsuddin 1983). While there are marked histological and ultrastructural similarities with human colonic adenocarcinomas there are also differences. The well-documented "adenoma-carcinoma" sequence in human disease is less clearly established, although evidence in favour of it occurring in this experimental model has been presented (Sunter *et al* 1978). More often carcinomas arise *de novo* in macroscopically flat mucosa (Shamsuddin 1983; Pozharisski 1975). The most significant deviation, however, from the human situation is the rarity of hepatic metastasis, a factor which does detract from this otherwise highly valuable model of colorectal cancer.

The hydrazine carcinogen model has been used as a standard means of inducing colorectal cancer in animals, allowing the influence of putative promoting factors, inhibitors and novel therapeutic strategies to be studied. Several groups have utilised this model to examine the influence of gastrin and their work is described next.

(ii) Studies of gastrin in hydrazine carcinogenesis

There have been relatively few studies involving gastrin and hydrazine-induced colorectal tumours in rodents. Although detailed and labour-intensive, such studies involve small numbers of animals in each treatment group and, as a result, demonstrating a statistically significant difference in tumour incidence, propensity for metastasis or survival has been difficult. Also, studies are difficult to compare because of differences in experimental design, species, strain, age and sex of animals used, methods employed to induce hypergastrinaemia and means of assessing tumour proliferation and growth.

Two other problems pertinent to studies of gastrin in experimental colorectal carcinogenesis deserve mention. Firstly, surgical methods of inducing hypergastrinaemia, for example by fundectomy or antral exclusion, may lead to a reduced gastric capacity with subsequent lower food and caloric intake, resulting in diminished weight gain compared to control animals. Calorie restriction with impaired weight gain has been shown to inhibit carcinogenesis in many different animal models

(Albanes 1987; Birt *et al* 1992) and this is discussed in more detail later (Chapter 9). Secondly, intestinal surgery produces non-specific tissue injury and enhancement of hydrazine-induced tumour formation at sites of surgical anastomoses and suture lines has also been documented (McGregor 1988). These potential confounding factors must be borne in mind when interpreting the results of animal carcinogenesis studies.

In an initial report, McGregor *et al* (1982) injected male Sprague-Dawley rats weekly with methylazoxymethanol for six weeks and then randomised them to undergo either antral exclusion (hypergastrinaemia), antrectomy (hypogastrinaemia), sham operation or sham plus pentagastrin injections (for forty-eight hours prior to death). When the animals were killed ten weeks later no difference in the number of tumours nor their distribution was observed among the groups. Mean tumour volume and total tumour "burden" were two-fold greater in hypergastrinaemic rats compared to normogastrinaemic animals although this was not statistically significant because of the wide range of individual values. Tumour contents of DNA, RNA and protein (measured by [^{3}H]thymidine, [^{14}C]orotic acid and [^{14}C]leucine uptake, respectively) were markedly higher in rats from the antral exclusion group as were calculated rates of DNA, RNA and protein synthesis. Similar results were observed in sham-operated rats which had received pentagastrin ($2mg.kg^{-1}$) for forty-eight hours prior to death, when compared to sham-only animals. In contrast antrectomy failed to produce a sustained fall in gastrin levels and results in this group did not differ from control animals.

As this study examined the effects of gastrin *after* tumour initiation had already occurred, the authors repeated the study except that rats underwent either antral exclusion or sham operation two weeks *before* carcinogen (1,2-dimethylhydrazine) was given (Karlin *et al* 1985). Antral exclusion elevated mean fasting gastrin concentrations by almost three-fold relative to sham controls and all 100% (n = 23) of this group developed colorectal tumours as opposed to 72.7% of the normogastrinaemic control animals (n = 22). Rats in the antral exclusion group had on average more tumours per rat (4.2 versus 1.8) and two-fold greater mean tumour weight and volume. From these two studies the authors suggested that gastrin may not only promote the growth of

established colorectal tumours but that hypergastrinaemia itself may predispose to tumour development.

At around the same time Oscarson *et al* tested the same hypothesis by performing either antrectomy or fundectomy on male rats, immediately after which a course of DMH injections was begun (Oscarson *et al* 1982). Although gastrins were only measured in three animals per group, antrectomy modestly reduced hormone levels while fundectomy raised them up to five-fold compared to "control" animals (which had not undergone any sham procedure). No difference in tumour incidence or mucosal content of DNA or RNA was seen and colonic villous weight and crypt depth were also similar in the two groups. These histological parameters are crude ways of assessing trophic effects and there were also considerable weight differences among the groups with fundectomised rats weighing on average one hundred grams less than control animals. This represented a weight difference of approximately 40% and may well have influenced the carcinogenic process. In another study antral exclusion raised fasting gastrin levels four-fold and significantly stimulated DNA content and the rate of DNA synthesis ([^{3}H]thymidine uptake) in both DMH-induced tumours and "normal" colonic mucosa, compared to sham-operated animals (Elwyn *et al* 1985). No difference in tumour number, size, weight or position was observed although each treatment group consisted of less than ten rats.

In an autoradiographic study two injections of pentagastrin ($120\mu g.kg^{-1}$) given to rats with DMH-induced colorectal tumours led to a 45% increase in the labelling index of cancer cells relative to control animals (Lamote and Willems 1988). The authors repeated the study and assessed tumour proliferation by a stathmokinetic technique and again pentagastrin was trophic to the cancer cells, increasing the number of vincristine-arrested metaphases (the metaphase index) by 108% compared to controls. From these results the authors concluded that pentagastrin increased both the proportion of cells in the DNA-synthesis phase (S-phase) of the cell-cycle and the number of these progressing to the mitotic phase (M-phase).

Given the recent controversial evidence that surgery for peptic ulcer may predispose to the late development of colorectal cancer (Bundred *et al* 1985), several

investigators have examined the effect of such surgery on chemical carcinogenesis in rats, with conflicting results (Houghton *et al* 1990; Oscarson *et al* 1982). While surgical procedures such as vagotomy and Billroth I partial gastrectomy induce endogenous hypergastrinaemia, several other important variables are also altered, for example acid secretion and bile acid metabolism. When the possible effects of altered calorie intake and weight gain are also considered it is difficult to draw clear conclusions about the relevance of gastrin to any observed changes in colonic cancer development in such studies. Similarly, while proximal small bowel resection in animals causes hypergastrinaemia, it has several other effects which could equally explain the enhancement of hydrazine-induced colorectal carcinogenesis produced by the operation (Williamson *et al* 1978; Tilson 1980).

(iii) Studies of gastrin in other models of chemical carcinogenesis

Apart from hydrazines, of the other colonic carcinogens described above, only N-methyl-N^1-nitro-N-nitrosoguanidine (MNNG) has been used in studies of the effects of gastrin on tumourigenesis. In one study depot injections of tetragastrin given during and after intrarectal MNNG treatment significantly *reduced* the development of colorectal tumours in rats (Tatsuta *et al* 1983). The authors confirmed these results in a further small study, showing that tetragastrin reduced tumour incidence and size when given from the start of MNNG treatment but had no effect if delayed until after MNNG dosing was complete (Tatsuta *et al* 1985). While some studies of gastrin and colorectal cancer have failed to demonstrate a stimulatory effect, these two papers are the only ones to record *inhibitory* effects of the hormone on tumourigenesis and the results have not been corroborated by other groups.

4.2.2. Xenograft Models of Colorectal Cancer

(i) Introduction

The availability of strains of genetically athymic 'nude' mice allows us to grow a wide range of tumours as subcutaneous xenografts in such animals without the problems of immune rejection. Notably, human tumours can be grown in nude mice, their growth can be closely followed and the effects of experimental interventions on tumour growth and animal survival can be quantitated. As such, this model has been extensively used to study the effects of gastrin on the growth of colorectal cancer cells.

(ii) Effects of gastrin on colorectal cancer xenografts

A trophic effect of gastrin on colorectal cancer *in vivo* was first noted by Svet-Moldavsky (1980). In a short report, repeated injections of pentagastrin ($250\mu g.kg^{-1}$) increased the weight of a mouse adenocarcinoma (AKATOL-1-71) twenty-fold compared to saline-treated control animals. Interestingly, a higher dose of pentagastrin ($600\mu g.kg^{-1}$) had a less pronounced effect on tumour growth. No effect was noted on other tumours, including a hepatoma and a small bowel adenocarcinoma. Two subsequent reports followed, the first finding no effect of pentagastrin on the growth or labelling index of a transplantable human colorectal cancer (Sumiyoshi *et al* 1984). In the second, one of three human colonic tumour xenografts did respond to pentagastrin with an increase in weight and a decrease in tumour doubling time (Tanaka *et al* 1986).

Around the same time investigators in Texas were studying a carcinogen-induced murine colorectal cancer (CT-26) and established it as a transplantable tumour in nude mice, since when it has been extensively characterised and shown to be gastrin-receptor positive and gastrin-responsive (Townsend *et al* 1990). Winsett *et al* (1985) found varying doses of pentagastrin stimulated growth and significantly increased DNA content in this tumour, with the maximal response to pentagastrin occurring at a lower dose in the tumours than in normal gastric fundic mucosa. In addition, pentagastrin-treated animals had significantly reduced survival at thirty-five days (55%) compared to saline-treated controls (90%). By day fifty-five all pentagastrin-treated animals were

dead whereas 80% of control mice were still alive. In a subsequent study the same group were able to prevent these effects of pentagastrin with the gastrin-receptor antagonist proglumide (Beauchamp *et al* 1985b) and this is discussed more fully later.

The trophic effects of pentagastrin on MC-26 tumours have been confirmed in a number of different studies (Singh *et al* 1986b, 1987, 1993b). Recently others repeated the study of the Texas group (Alonso *et al* 1992) and confirmed the growth-stimulatory effects of gastrin, with significant increases in mean tumour weight (40%), volume (55%) and DNA content (30%). Mean survival in pentagastrin-treated mice was also significantly shortened (29.6 days) relative to control animals (42.5 days).

Initial investigations by Watson *et al* found that while gastrin stimulated growth of a human gastric cancer xenograft (MKN45), repeated injections of gastrin had no effect on HT29 colorectal cancer cells (Watson *et al* 1988). When gastrin was administered as a continuous infusion by osmotic minipump, however, a trend towards increased growth of HT29 cells was observed although statistical significance was not reached. Using the same technique in a later study, the authors reported significant growth stimulation of another human colonic cancer cell line (C523) grown as xenografts (Watson *et al* 1992). It may, therefore be that continuous exposure of tumour cells to relatively constant low concentrations of gastrin may be more relevant than repeated bolus injections at high doses and at relatively long dosage intervals.

Several other authors have reported on the effects of gastrin on a variety of different human colorectal cancer cells grown as xenografts in nude mice with variable and sometimes conflicting results (Smith *et al* 1988, 1993; McGregor *et al* 1989; Eggstein *et al* 1992). As discussed in previous sections methodological differences are likely to account for much of this. The results of all of these, along with other published experimental studies, are summarised in Table 4.1. It should be noted that a number of other 'negative' studies which have not been published are likely to exist.

Of all the studies on gastrin and colorectal cancer growth in animals, only one has used pharmacologically-induced endogenous hypergastrinaemia as opposed to surgical methods or exogenous gastrin. In this paper mice were inoculated with MC-26 cells and were dosed with omeprazole or buffer by oral gavage for nineteen days

(Graffner *et al* 1992). Although omeprazole-treated animals had five-fold elevated median serum gastrin levels compared to controls, no effect on tumour size nor animal survival was noted. The mice were inoculated with large numbers of cancer cells, however, resulting in rapidly growing tumours and death occurred very quickly in both groups. It is possible, therefore, that this made differences in tumour growth and survival difficult to detect.

The xenograft model has provided much useful information to compliment that derived from other animal studies and *in vitro* systems. While methodological differences have to be borne in mind, such experiments have shown that large doses of gastrin are capable of stimulating the growth of colorectal cancer *in vivo* and may also affect animal survival.

Xenograft	Origin	Hormone	Dose Range	Duration	Growth parameters	Response	Reference
Co-3	human	PG	$10\mu g.day^{-1}$	28 days	tumour wt., DNA content & L.I.	–	Sumiyoshi *et al* 1984
ACL-1, ACL-5, ACL-6	human	PG	$250\mu g.kg^{-1}$	4-5 weeks	tumour wt. & doubling time (T_D)	+ (ACL-1)	Tanaka *et al* 1986
LoVo	human	G-17	$10\mu g.day^{-1}$ or $0.4\text{-}10\mu g.day^{-1}$ (osmotic pump)	14 days	tumour cross-sectional area	+/- (pump)	Watson *et al* 1988
C523	human	G-17	$10\mu g.day^{-1}$ (osmotic pump)	17 days	tumour cross-sectional area	+	Watson *et al* 1992
CX1	human	PG	$0.5\text{-}1.0mg.kg.^{-1}day^{-1}$	14-25 days	tumour wt., vol., DNA & protein content	+	Smith *et al* 1988
X56	human	PG	$0.5\text{-}1.0mg.kg.^{-1}day^{-1}$	14-25 days	tumour wt., vol., DNA & protein content	+	Smith *et al* 1988
WiDr	human	PG	$1mg.kg.^{-1}day^{-1}$	14 days	tumour vol., DNA & protein content	+	Smith *et al* 1993
WiDr	human	PG	$2mg.kg.^{-1}day^{-1}$	14 days	DNA, RNA & protein content	+	McGregor *et al* 1989
COLO 320DM	human	PG	$2mg.kg.^{-1}day^{-1}$	14 days	DNA, RNA & protein content	+	McGregor *et al* 1989

Table 4.1 (2 pages). Published studies of gastrin in animal models of colorectal cancer.
PG = pentagastrin, G-17 = gastrin-17. + trophic effect, - no effect. L.I. = labelling index.

Xenograft	Origin	Hormone	Dose Range	Duration	Growth parameters	Response	Reference
"fresh sigmoid adenocarcinoma"	human	PG	2mg.kg.$^{-1}$day^{-1}	14 days	DNA, RNA & protein content	+	McGregor *et al* 198
SW403	human	PG	60μg b.d.	28 days	ODC activity	+	Eggstein *et al* 1991
LS174T	human	PG	60μg b.d.	28 days	ODC activity	_	Eggstein *et al* 1991
AKATOL-1-71	mouse	PG	250 or 600μg.kg^{-1}	16-25 injns.	tumour wt.	+	Svet-Moldavsky 1980
MC-26	mouse	PG	125-500μg.kg^{-1} t.i.d.	14 days	tumour wt. & DNA content; survival	+	Winsett *et al* 1985
MC-26	mouse	PG	250μg.kg^{-1} t.i.d.	7-15 days	tumour wt.	+	Singh *et al* 1986
MC-26	mouse	PG	250μg.kg^{-1} t.i.d.	21 days	tumour wt.	+	Singh *et al* 1987;1993
MC-26	mouse	(see text)	omeprazole 400μmol.kg.$^{-1}$day^{-1}	19 days	tumour wt.; survival	_	Graffner *et al* 1992
CT-26	mouse	PG	250μg.kg^{-1} t.i.d.	14 days	tumour wt., volume, DNA content; survival	+	Alonso *et al* 1992

Table 4.1 contd. (page 2 of 2).

CHAPTER 5

GASTRIN/CCK-B RECEPTORS IN COLORECTAL NEOPLASIA

5.1 INTRODUCTION

Like other gastrointestinal peptides, gastrin exerts its biological actions by interacting with specific cell surface receptors. If gastrin is of relevance to human colorectal cancer, then identifying and characterising gastrin receptors on tumour cells is of crucial importance before gastrin receptor antagonists are to be considered as therapeutic options in this disease.

For nearly twenty years the binding of radiolabelled biologically active gastrin (or agonists of gastrin) to tissues of interest has been used to identify and characterise gastrin receptors. Analysis of binding data enables the number of binding sites and dissociation constant (K_d) to be calculated (Kleveland and Waldum 1991; Bylund and Yamamura 1990). Using this method specific receptors are generally considered to be present if binding is specific, saturable and restricted to tissues known to be biologically regulated by the hormone of interest. Furthermore, the K_d should be similar to the plasma concentrations of peptide seen physiologically and certain other kinetic criteria should also be fulfilled. Lastly, and perhaps most importantly, the observed binding should result in a biological response although this has often been difficult to demonstrate, especially with respect to gastrin's trophic effects on neoplastic cells.

A great deal has been published on gastrin receptors in the gastric mucosa and their role in the control of acid secretion and this is reviewed elsewhere (Chaung *et al* 1993). Before discussing evidence pertaining to gastrin receptors in colorectal cancer, several points deserve emphasis. Firstly, the radioligand binding assay itself is not without problems, depending on the presence of a stable gastrin tracer which retains biological activity, a suitable *in vitro* tissue preparation and suitable incubation conditions. Early methods of iodinating gastrin resulted in loss of biological activity although this has been overcome in recent years. The procedure depends on tissue homogenisation, thus making cellular localisation of receptor-binding impossible in tissues composed of heterogeneous cell types. Homogenisation itself may result in loss of receptors especially through release of proteolytic enzymes and, depending on the

exact incubation conditions, incubation damage can occur during the binding assay itself with loss of label and/or receptors.

The second point concerns the identification, characterisation and classification of "gastrin receptors". Gastrin and cholecystokinin (CCK) are the key members of a peptide family which share the common carboxyl terminal sequence (-Gly-Trp-Met-Asp-Phe.NH_2). The receptors which bind gastrin and CCK have traditionally been classified according to their relative affinities for different agonists (Presti and Gardner 1993; Hughes *et al* 1993). CCK-A ("alimentary") receptors have much higher affinity for CCK than gastrin whereas CCK-B ("brain") receptors bind CCK with only slightly greater affinity than gastrin. Sulphated CCK has much greater affinity for receptors than non-sulphated hormone whereas sulphation appears to make little difference to the binding of gastrin (Huang *et al* 1989). The existence of a third class of distinct gastrin receptors (CCK-C or CCK-G), which bind CCK and gastrin with equal affinity is debated (Bold *et al* 1994). Overall considerable interspecies variation (and even tissue variation with a given species) seems to exist and current evidence suggests that CCK-B and gastrin receptors are pharmacologically identical within a species, although they may be linked to different G-proteins (Hughes *et al* 1993). It is likely that a conclusive answer to this question will depend on further analyses at the molecular level (discussed later) but from this point on the terms "gastrin receptor" and "CCK-B receptor" are considered to represent the same receptor and referred to as the "gastrin/CCK-B" receptor. The possibility that different affinity states for the same receptor (high and low) may co-exist in the same tissue should be borne in mind, as should the possibility that malignant tumours may express different or abnormal receptors compared to their normal tissue of origin.

5.2 GASTRIN/CCK-B RECEPTORS AND CANCER

5.2.1 Colorectal Cancer

Although specific binding of gastrin to receptors in rat gastric mucosa had been demonstrated as early as 1976 (Lewin *et al* 1976) and the receptor assay methodology described in 1979 (Takeuchi *et al* 1979), the first major demonstration of gastrin receptors on colonic epithelial cells was not published until 1985 (Singh *et al* 1985). This group, from Galveston in Texas, has subsequently produced many studies on the role of gastrin receptors in colon cancer. In their initial detailed study, high-affinity gastrin receptors (K_d 0.4-0.55nM) were found on crude membrane fractions from normal rat colonic mucosa. High-affinity (K_d = 0.25-0.6nM) sites were also identified on membranes from one human (LoVo) and one murine (MC-26) colon cancer cell line whereas two other human cell lines (HT29 and HC845) showed little or no binding (Singh *et al* 1985). Furthermore the binding affinities for the colon cancer cell lines and normal rat gastric mucosa were similar. Subsequent studies using the MC-26 model confirmed the presence of gastrin receptors in this tumour (Singh *et al* 1986b, 1987, 1993; Chicone *et al* 1989; Guo *et al* 1990). These reports demonstrated not only that the trophic effects of gastrin were mediated via gastrin receptors, but also suggested that gastrin was necessary to maintain both receptor numbers and their binding affinity as the tumours grew. Thus, tumours in rats which were not pentagastrin-treated lost their high-affinity gastrin receptors as they progressed (Singh *et al* 1987). This supports the earlier work of Takeuchi *et al* (1980) suggesting that gastrin "upregulates" its own receptor possibly by stimulating production of the receptor.

A number of other authors have also examined the presence of gastrin receptors in colorectal cancer cells with variable results. Once again differences in assay methodology, use of long-established cell lines and species differences etc., which have been highlighted in preceding sections, probably apply to studies of gastrin receptors and these points need to be borne in mind when interpreting the results of these papers. For example, the effects of cell "synchronisation" on the trophic effects of gastrin *in vitro* (discussed in Chapter 3) could be explained by changes in gastrin receptor

numbers. Guo *et al* found that gastrin receptor content in synchronised MC-26 cells was three-fold greater than in asynchronous cells (Guo *et al* 1990). It should also be noted that some workers have only identified gastrin binding of low-affinity, with K_d's in the micromolar or millimolar range and the significance of such receptors is as yet unclear (Weinstock and Baldwin 1988).

Frucht *et al* found gastrin binding on only one of ten human colonic cancer cell lines, although six of these lines were derived from secondary deposits and not the primary tumour (Frucht *et al* 1992). Furthermore, only one concentration of gastrin was used (100pM) in the binding studies rather than a range of concentrations. Finally, CCK binding was observed in three of the ten lines but as competition assays with CCK receptor antagonists were not performed, it is unclear whether the binding represented CCK-A or gastrin/CCK-B receptors. While several reports have demonstrated the *in vitro* gastrin-responsiveness of fresh human colorectal cancers (Watson *et al* 1989a) as an indirect marker of gastrin receptor status, very few have directly demonstrated high-affinity gastrin binding. Again it is likely that more authors have examined this but not published their negative results. High-affinity (K_d = 0.4-0.6nM) gastrin binding sites on fresh primary human colorectal tumours were first reported (in abstract form) in 1981 (Rae-Venter *et al* 1981). Partially purified membranes from seven of eight human tumours contained gastrin receptors albeit at low capacity (0.5 $fmol.mg^{-1}$ membrane protein). Receptors were also found in three samples of normal colorectal mucosa taken at colonoscopic biopsy, although little data about assay methodology is available from their brief report.

The same group, from the University of Texas at Galveston, then published the results of a more detailed study in 1989 (Upp *et al* 1989). Specimens of colorectal tumours and "healthy" colonic mucosa were taken from freshly resected tumours and gastrin receptor assays performed on crude membrane fractions. From their results, high affinity receptors (K_d < 1nM) were observed on thirty-eight of sixty-seven cancers (56.7%) with a further seven tumours found to have only low-affinity gastrin binding (K_d > 1nM). Positive tumours exhibited only a single class of binding sites with K_d's of 0.1-0.3nM and the GR content varied widely from 1.5-50$fmol.mg^{-1}$ membrane protein.

Twenty of the thirty-eight people with GR-positive tumours had GR contents above 10fmol.mg^{-1}. No correlation with patient age, sex, CEA level nor degree of differentiation was found but the mean GR content of Dukes' A or B tumours was twice that of Dukes' Stage C or D lesions. Similar high affinity GR were found in the "normal" mucosa in twenty-eight of fifty-nine samples available (47.5%) with low affinity binding seen in nine more cases. Twenty-two (37%) of samples had no detectable gastrin receptors and there was a highly significant correlation between the presence of GR on "normal" mucosa and on tumours, although it has to be remembered that while microscopically disease-free, this mucosa cannot be considered truly normal. Other studies by these authors have also identified high affinity GR in membranes prepared from freshly-resected human colorectal tumours (Upp *et al* 1988; Chicone *et al* 1989). In contrast, in one small study, Kumamoto *et al* were unable to demonstrate significant gastrin receptors in "normal" colonic mucosa from two patients undergoing colorectal cancer surgery even though such receptors were found in gastric fundic and duodenal mucosa (Kumamoto *et al* 1989). Unfortunately no data was presented on the colorectal tumours themselves.

5.2.2 Gastrin Receptors in Other Tumours

Detailed discussion of the presence and role of gastrin/CCK-B receptors outwith the colorectum is beyond the scope of the present work. In brief, many studies have identified high affinity gastrin receptors on membranes from human and rodent gastric (Singh *et al* 1985; Weinstock and Baldwin 1988; Watson *et al* 1992; Ishizuka *et al* 1992) and pancreatic (Scemama *et al* 1987; Watson *et al* 1991a; Zhou *et al* 1992; Soundararajan *et al* 1993) carcinomas. They may also be also present on enterochromaffin-like cells (ECL-cells) from gastric carcinoid tumours which occur spontaneously in *Mastomys natalensis* animals (Reubi *et al* 1992; Inomoto *et al* 1993).

Outwith the gastrointestinal tract CCK-B receptors have been identified on several human small cell lung cancer cell lines (Yoder and Moody 1987; Sethi and Rozengurt 1992). Increasing attention is being paid to interactions between the immune

and neuroendocrine systems and it is of interest that CCK receptors with B-type pharmacology have been demonstrated on a human T-cell lymphoblastic cell line (Lignon *et al* 1991).

5.2.3 Molecular Characterisation of Gastrin/CCK-B Receptors

Very little is currently known about the molecular basis of gastrin-binding proteins so far identified on normal and malignant colonic epithelium. The likelihood of species-dependent and tissue-tissue variations in receptor structure, signalling and function make extrapolation from available data potentially misleading.

Using covalent cross-linking and protein electrophoresis, Chicone *et al* have reported that gastrin binds predominantly to a single peptide of 33-40 kDa in membranes of both MC-26 and fresh human colorectal tumours (Chicone *et al* 1989) but this protein has yet to be sequenced. Alternatively, a monoclonal antibody raised to canine parietal cells, and which recognises a 78 kDa gastrin binding protein in porcine stomach, also binds to cell membranes in human colorectal tumours (Watson *et al* 1993).

Since 1992 the gastrin/CCK-B receptor has been cloned and sequenced from a variety of sources, including rat brain and pancreas (Wank *et al* 1992), human brain and stomach (Pisegna *et al* 1992; Miyake *et al* 1994), canine parietal cells (Kopin *et al* 1992) and ECL carcinoid tumours from *Mastomys natalensis* (Nakata *et al* 1992). A high degree of nucleic acid sequence homology among these isolates is apparent but whether gastrin/CCK-B receptors are identical in different tissues within the same species is not yet entirely clear. Recent evidence suggests that they are the same (Lee *et al* 1993; Miyake *et al* 1994), although Yamada's group have suggested, on the basis of the molecular structure of the human gastrin receptor gene, that alternative splicing pathways yielding receptor variants may exist (Song *et al* 1993). Preliminary data published in 1994 has raised the possibility that gastrin receptors in colon cancer may be distinct from classical CCK-B receptors (Bold *et al* 1994). In this study CCK-B receptor mRNA was not detected in LoVo cells by Northern hybridisation and the

trophic effects of gastrin could not be blocked by the CCK-B antagonist L365,260. The same group have also reported, however, that another selective CCK-B antagonist (JMV 320) *does* inhibit growth of these cells (Ishizuka *et al* 1994). Further rapid developments in this field will hopefully shed light on the exact nature of the gastrin receptor on colonic and other gastrointestinal tumours.

5.3 CHOLECYSTOKININ AND GASTRIN RECEPTOR ANTAGONISTS

5.3.1 Introduction

For many years there were few antagonists available for use in studies of gastrin/CCK-B receptors and some of these were weak with low affinity for receptors. Recently, however, many new classes of peptide and non-peptide antagonists have been described, resulting in rapid advances in our knowledge of CCK receptor classification and pharmacology. As yet few of these newly available gastrin/CCK-B receptor antagonists have been used in studies of colorectal cancer although results of their use in this area are likely to be forthcoming and are keenly awaited. At least eight classes of CCK receptor antagonists have been described (Jensen *et al* 1990; Presti and Gardner 1993) and within each class are compounds with sufficient selectivity to distinguish between CCK-A and gastrin/CCK-B receptors.

5.3.2 Early Studies Using Proglumide

The earliest studies of gastrin receptor antagonists in experimental colonic cancer utilised the glutaramic acid derivative proglumide ((±)-4-(benzolyamino)-5-(dipropylamino)-5-oxopentanoic acid). While this compound had been shown to be a specific, competitive inhibitor of gastrin-stimulated gastric acid secretion in the late 1960's, its effects on growth in the gastrointestinal tract were not reported until 1984 (Johnson and Guthrie 1984). In this study, high doses of proglumide partially inhibited pentagastrin-stimulated DNA synthesis and content in scrapings of normal rat colonic mucosa. Proglumide had no effects when used on its own and the IC_{50} was in the

millimolar range. Other authors have also found proglumide to inhibit gastrin or pentagastrin-stimulated growth of colon cancer cells both *in vivo* and *in vitro* (Singh *et al* 1986a, 1987; Smith *et al* 1988; Yactayo *et al* 1991). In none of these studies did proglumide inhibit the basal growth of tumour cells although this was noted in several other *in vitro* studies using a variety of colorectal cancer cell lines (Hoosein *et al* 1989, 1990; Guo *et al* 1990).

Following up their earlier study which found that pentagastrin enhanced growth of MC-26 tumours and reduced survival in nude mice (Winsett *et al* 1985), the same group examined the effects of proglumide in this model (Beauchamp *et al* 1985b). Compared to saline-treated control rats, proglumide treatment significantly reduced growth, DNA and RNA content in both tumours and in normal colonic mucosa. By day twenty-one of treatment mean tumour size was only 46% of control and, of more interest, proglumide increased mean survival from 25.3 days in control mice to 39.2 days.

Not all authors have been able to demonstrate a growth inhibitory effect of proglumide on colorectal cancer cells (Romani *et al* 1994) and in some studies where effects were noted, the effective doses for proglumide were often in the micromolar to millimolar range. Whether such observed effects are of physiological relevance is questionable. In addition one recent study found that while proglumide reduced the growth of gastrin-responsive HT29 cells, it also inhibited the growth of fibroblastic 3T3 cells *in vitro* (Mauss *et al* 1994) and similar observations have been made by others (Piontek *et al* 1993). These cells show no gastrin binding, are not trophically stimulated by gastrin and the effects of proglumide could not be reversed by adding large concentrations gastrin, suggesting that proglumide's actions may be non-specific and not due to gastrin-receptor binding. Clinically, the drug has only been used in one small study of patients with advanced colorectal cancer and, perhaps not surprisingly, no effects on patient survival or tumour growth were found (Morris *et al* 1990).

5.3.3 Other Gastrin/CCK-B Receptor Antagonists

Along with proglumide several early investigations used benzotript, a derivative of the amino acid tryptophan. This compound also has low affinity for gastrin/CCK-B receptors with a K_d in the millimolar range. Using doses of 0.4-1.6mM, Hoosein *et al* (1989) observed inhibitory effects on monolayer growth and anchorage-independence using six different human colorectal cancer cell lines. The IC_{50} for gastrin receptor binding was 0.4mM, however, and it would be almost impossible for the drug to achieve concentrations *in vivo* sufficient to cause effective receptor blockade. Similar inhibitory effects on the growth of murine MC26 colon cancer cells *in vitro* have been reported (Guo *et al* 1990). Eggstein *et al* (1991) found that benzotript only inhibited the growth of a gastrin-responsive colonic cancer cell line (SW403) but had no effect on cell lines whose growth was not stimulated by gastrin and concluded that the antagonist's actions were specifically related to its receptor-blocking properties.

More recently several compounds of varying chemical structure have been developed as highly selective and potent antagonists for gastrin/CCK-B receptors. The benzodiazepine compound L365,260 is one of the most potent (Presti and Gardner 1993; Hughes *et al* 1993) and in one study the *in vitro* basal growth rates of two human colorectal cancer cell lines (LoVo and C146) were reduced to 44% and 64% of control cells, respectively (Watson *et al* 1991). Once the drug was removed, however, growth of the cancer cells rapidly returned to control levels. In contrast L365,260 was reported to have no effect on the growth of two other human colorectal cancer cell lines (Thumwood *et al* 1991).

Using the newer non-peptidic glutamic acid derivative CR2093 (R-4-(3-chlorobenzamido)-5-(3,3-dimethyl butylamino)-5-oxo-pentanoic acid), Watson *et al* examined its effects on the growth of various gastrointestinal tumour cell lines, both *in vitro* and when grown as xenografts in nude mice (Watson *et al* 1992). High affinity gastrin/CCK-B receptors were demonstrated on C523 cells (K_d 2.2 x 10^{-10}M) *in vitro* and the IC_{50} was in the millimolar range (>10^{-4}M). Continuous intravenous infusion of the antagonist in doses sufficient to inhibit gastric acid secretion inhibited neither basal

nor gastrin-stimulated growth of C523 xenografts. Conversely, the growth of AR42J (rat pancreatic) cells and MKN45 (human gastric) cells was inhibited. These tumours, however, possessed lower affinity receptors and the authors suggested that the higher the receptor affinity for gastrin, the greater the concentration of antagonist needed to compete effectively and displace gastrin. Thus it is possible that higher doses may have had an effect on the colorectal cancer cells.

The highly selective and potent gastrin/CCK-B receptor antagonist JMV 320 has been found in one study to inhibit the gastrin-stimulated growth of several human colorectal cell lines *in vitro* without having any effect when given alone (Ishizuka *et al* 1994).

Very recently, studies of newly-developed gastrin receptor antagonists have been reported in abstract form. In the first, neither M224624 nor M226770 was able to inhibit the growth of either MC26 (mouse) or C523 (human) colon cancer xenografts when given in the form of slow-release depot injections (Garner *et al* 1992). In the second report, the *in vitro* basal growth of four out of six human colonic carcinoma cell lines was reduced to 30% of control by the highly potent gastrin/CCK-B receptor antagonist C1988 (Romani *et al* 1994). The CCK-A and CCK-B antagonist CAM 1481 was also effective and the drugs were said to exert activity at "doses as low as 10^{-11}M" suggesting physiological relevance, although few details are currently available. When one of the cell lines (LoVo) was grown as a xenograft, C1988 inhibited its *in vivo* growth by 53% when given orally for twenty days.

5.3.4 Studies Involving Antigastrin Antibodies

An alternative approach to the use of pharmacological receptor antagonists is to use specific antigastrin antisera and this technique is currently being evaluated. In 1989 Hoosein *et al* reported inhibition of the basal growth of two human colon cancer lines by a polyclonal antigastrin antibody (Hoosein *et al* 1989). Non-immune rabbit IgG had no effect and pre-incubation of the antibody with G-17 abolished its activity. A later study by the same group confirmed these findings in a number of other colorectal cell

lines (Hoosein *et al* 1990). A polyclonal antiserum (ICI-925), selective for the N-terminal region of gastrin, was able to "markedly inhibit" the growth of C523 human colon cancer xenografts when infused continuously into nude mice at a dose sufficient to abolish gastric acid secretion (Garner *et al* 1992). Curiously, however, a monoclonal antibody (Cure Gas -93) was without effect.

The Nottingham group have developed a novel approach to raise antigastrin antibodies *in vivo* by immunising animals with the amino terminus of G-17 linked to diphtheria toxoid. The immunogen (called "Gastrimmune") induces specific anti-G-17 antibodies which do not cross-react with either G-34 or CCK (Watson *et al* 1993, 1994). In experimental animal models, these authors have found Gastrimmune treatment to inhibit significantly the growth of gastrin-receptor positive colonic cancers, effects presumed to operate via inhibition of the trophic effects of endogenous gastrin. More detailed reports are awaited and a clinical trial in patients with advanced colorectal cancer is currently underway (S. Watson, personal communication).

5.4 SUMMARY

It seems likely that a significant proportion of human colorectal tumours possess high-affinity "gastrin-binding sites", although the functional relevance of these has not been conclusively shown. Whether such gastrin-binding sites are identical or not to classical gastrin/CCK-B receptors in other tissues remains to be settled, although they probably are. Early studies involving weak, relatively non-specific receptor antagonists should be interpreted with caution and further studies using newly available, more potent compounds are needed. Finally, early results of studies involving antigastrin antibodies raised by a synthetic immunogen appear promising and further evaluation is awaited.

CHAPTER 6

POST-RECEPTOR SIGNALS MEDIATING THE TROPHIC EFFECTS OF GASTRIN

6.1 POST-RECEPTOR SIGNALLING

The mechanisms mediating signal transduction following binding of gastrin to its receptor in the colon are poorly understood as are the exact intracellular processes stimulated in the proliferative response to the hormone. While much has been discovered about the post-receptor signals involved in the acid-secretory response of gastric parietal cells to gastrin (Yamada *et al* 1993) this may not be relevant to the colon as the two receptors have not yet been conclusively shown to be identical. Even if they are the same their regulation may differ in different tissues, for example, by coupling to different G-proteins.

In gastric parietal cells, gastrin stimulates turnover of inositol phospholipids, culminating in elevation of intracellular calcium and activation of protein kinase C (PKC). PKC also seems to activate an autoregulatory mechanism resulting in receptor down-regulation with reduced receptor numbers (Yamada *et al* 1993). Evidence presented by Yassin *et al* suggests that gastrin does stimulate PKC activity in isolated normal rat colonocytes (Yassin *et al* 1991a). In addition the effects of gastrin could be abolished by either proglumide or staurosporine, an inhibitor of PKC (Yassin *et al* 1993). These authors also demonstrated that gastrin at an optimum dose of 10nM induced the transcription of a wide range of mRNA species in these cells and markedly stimulated protein synthesis (Yassin *et al* 1991b). This was also abolished by staurosporine. Coupling of gastrin/CCK-B receptors to PKC activation also occurs in the rat pancreatic cancer cell line AR42J (Seva *et al* 1994b).

As PKC activity is closely related to stimulation of membrane phosphotidylinositol (PI) hydrolysis, studies have now begun to examine this aspect of the response of colon cancer cells to gastrin . In a recent detailed study, gastrin at doses of 10^{-10}-10^{-12}M stimulated the growth of several human colorectal cancer cell lines and in one of these lines this was associated with a parallel stimulation of PI hydrolysis and intracellular calcium mobilisation (Ishizuka *et al* 1994). These effects were blocked by the potent and selective gastrin/CCK-B receptor antagonist JMV 320. Interestingly, the other cell lines responded to gastrin with an increase in cAMP turnover but without any

change in PI or intracellular calcium. It would appear, therefore, that gastrin signalling in colonic cancer cells may occur through several different mechanisms.

The same authors have previously found that gastrin stimulates mobilisation of intracellular calcium independently of phosphotidylinositol hydrolysis in a gastric carcinoma cell line (AGS) which possesses gastrin receptors and responds trophically to the hormone (Ishizuka *et al* 1992). In addition, the hormone had no effect on intracellular calcium in a subclone lacking gastrin receptors. Similar effects of gastrin on growth and calcium mobilisation have been observed in small cell lung cancer cells (Sethi and Rozengurt 1992) and in NIH3T3 fibroblasts expressing the cloned human gastrin/CCK-B receptor (Taniguchi *et al* 1994).

Regulation of cellular proliferation is thought to depend on a readily available supply of polyamines although the exact roles of these ubiquitous compounds are not fully understood. Activity of the enzyme ornithine decarboxylase (ODC), which converts ornithine to putrescine, is the rate-limiting step and an increased level of ODC is one of the earliest steps in cellular proliferation (reviewed in Johnson *et al* 1993). High levels of polyamines and increased ODC activity occur in colorectal tumours (Kingsnorth *et al* 1984; Lamuraglia *et al* 1986). In several parts of the gastrointestinal tract, including the colon, exogenous gastrin stimulates ODC activity and increases mucosal polyamine content in parallel with its trophic effects (Seidel *et al* 1985; Arlow *et al* 1990; Majumdar 1990). Similarly endogenous hypergastrinaemia, induced by daily treatment with omeprazole, increased rat colonic polyamine content by 74% compared to control animals and this was associated with an increase in crypt cell production rate (Gray *et al* 1993). Others have also found gastrin to stimulate increased polyamine turnover in both colonic cancer cell lines and xenografts (Eggstein *et al* 1991; Smith *et al* 1993). In a study of freshly resected colorectal carcinoma specimens, Upp *et al* reported higher levels of polyamines in colorectal cancers which were gastrin receptor-positive than in tumours which did not possess gastrin receptors (Upp *et al* 1987).

The specific, irreversible inhibitor of ODC, α-difluoromethylornithine (DFMO) has been useful in studying the role of polyamines in cell proliferation. Several studies have found DFMO to inhibit the proliferative effects of gastrin on the colon, both *in*

vivo and *in vitro* (Seidel *et al* 1985; Majumdar 1990; Eggstein *et al* 1991; Smith *et al* 1993).

Thus, although polyamines would appear to be involved in the trophic response to gastrin, much remains to be learned about the cellular events occurring prior to this and also subsequently at the level of gene expression. In the study of Taniguchi *et al* (1994) noted above gastrin-signalling was associated with tyrosine kinase activation and increased expression of the proto-oncogenes *c-myc* and *c-fos* which are involved in the early response of cells to mitogenic stimuli. Pentagastrin has also recently been reported to stimulate expression of *c-myc* in cultures of normal small intestinal crypt cells (Wang *et al* 1993). The effects of gastrin on proto-oncogene expression in normal and neoplastic colorectal cells has not been reported to date but clearly merits investigation.

CHAPTER 7

GASTRIN AND HUMAN COLORECTAL NEOPLASIA

7.1 INTRODUCTION

What is the relevance of gastrin to colorectal neoplasia in humans ? Hypergastrinaemia occurs in a number of clinical states (Table 7.1) but little is known about whether any predisposition to colonic tumour formation occurs in these conditions.

In pernicious anaemia fasting circulating gastrin concentrations may be elevated forty to fifty-fold (Lamers 1980; Lanzon-Miller *et al* 1987a). There is wide inter-individual variation in the gastrin levels seen in pernicious anaemia patients, however, with some having levels more than one hundred-fold greater than normal people. While this condition is established as an important risk factor for subsequent gastric cancer development (Elsborg and Mosbech 1979) effects on colorectal cancer development are less clear. While a trend towards an increased risk of colorectal cancer was observed by Talley *et al* in a small population-based cohort study (Talley *et al* 1989), others have found no such association (Elsborg and Mosbech 1979; Brinton *et al* 1989).

Surgery for peptic ulcer disease, namely vagotomy and Billroth I gastrectomy (but not Billroth II surgery) also causes sustained rises in circulating gastrin levels (Blair *et al* 1986; Yamashita *et al* 1993). Several epidemiological studies have reported a long term increased risk of colorectal cancer development after peptic ulcer surgery (Caygill *et al* 1987; Stemmermann *et al* 1991), although this is still controversial (Kune *et al* 1988; Macintyre and O'Brien 1994). The association may be a complex one as factors other than hypergastrinaemia may be operative including reduced gastric acidity, altered bile acid metabolism, changes in intestinal flora and altered calcium metabolism. Interestingly the increased risk noted in the study of Caygill *et al* (1987) was restricted to females treated by either vagotomy or Billroth I gastrectomy (hypergastrinaemia) but not Billroth II gastrectomy (where no rise in gastrin occurs). The results of these retrospective studies need to be investigated further although prospective studies may not be feasible and would possibly take twenty years or more to yield an answer.

Worldwide, gastric acid antisecretory drugs (principally H_2-antagonists and H^+/K^+ ATPase inhibitors) are among the most widely prescribed of all drugs with an

Associated with gastric hypoacidity/achlorhydria

pernicious anaemia

atrophic gastritis

gastric surgery (Billroth I gastrectomy; vagotomy)

treatment with gastric acid-suppressing drugs (H_2-antagonists, H^+K^+-ATPase inhibitors)

Associated with gastric acid hypersecretion

Helicobacter pylori infection

antral G-cell hyperplasia

Zollinger-Ellison syndrome

chronic renal failure

gastric outlet obstruction

short bowel syndrome/small intestinal resection

Table 7.1. Clinical conditions associated with hypergastrinaemia.

estimated 1.7 million prescriptions in Scotland in 1993 alone (Dr. P. Rutledge, Medical Prescribing Adviser, Lothian Health Board - personal communication). These drugs indirectly cause sustained hypergastrinaemia consequent upon their inhibition of acid secretion and the more profound the effect on acid secretion the greater the rise in gastrin levels (Lanzon-Miller *et al* 1987b; Pounder 1993). While the majority of patients treated with omeprazole have two to four-fold elevations in fasting gastrin, there is marked inter- and intra-individual variation and in one study eight of thirty-two patients treated for two years or more had concentrations more than ten times the median level (Jansen *et al* 1990). Levels do return to normal soon after cessation of drug therapy and are unlikely to be of clinical relevance when used in short courses; the potential concerns relate to their longterm use in the management of acid-related disorders. Even though these drugs have been extensively investigated and prescribed for millions of people over the last ten to twenty years, this is still a relatively short period of time and possibly too early to allow detection of subtle but important effects on the trophic state of the gastrointestinal epithelium including that of the colon. This is supported by short term animal studies in which omeprazole-induced hypergastrinaemia was shown to increase the labelling index (vincristine-arrested metaphases) in normal rat colon (Pawlikowski *et al* 1991). Using the crypt cell production rate (CCPR), regarded by many as the most reliable method of assessing cell proliferation, Gray *et al* also found omeprazole to cause a small but statistically significant increase in rat colonic CCPR as well as marked stimulation of polyamine turnover (Gray *et al* 1993). In contrast to this, one small study found no significant increase in colonic proliferative markers in eight patients with Barrett's oesophagus treated with omeprazole sufficient to cause hypergastrinaemia (Murphy *et al* 1993). The subjects, however, were not 'normal', the technique used (*in vitro* [^{3}H]thymidine uptake into biopsies) is prone to inaccuracies, the rectum may not reflect other areas of the colon and there was marked interindividual variation noted.

At first glance patients with Zollinger-Ellison syndrome (ZES) could provide an attractive model for studying the effects of chronic endogenous hypergastrinaemia on the human colon. The rarity of the condition, however, and the fact that nearly all

such patients will either have had gastric surgery or be taking omeprazole makes it difficult to draw conclusions from this group of patients. In one small study Sobhani *et al* found that the labelling index of colonic biopsies (as measured by *in vitro* bromodeoxyuridine labelling) was significantly higher in ZES patients compared to healthy controls. No correlation was noted, however, between individual labelling indices and plasma gastrin concentrations (Sobhani *et al* 1993).

Only two small studies have so far examined a possible association between *H. pylori* infection and colorectal neoplasia and these have yielded opposite results (Lambert *et al* 1993; Justin *et al* 1994). Neither study was of sufficient size to answer this question adequately and both used serology alone to diagnose the presence of infection, which has a lower positive predictive value than other methods. Much larger, prospective, longitudinal studies would be required to address this issue fully.

7.2 Circulating Gastrin Concentrations and Human Colorectal Neoplasia

Once gastrin had been proposed as a growth factor for colon cancer cells *in vitro* and in animal models, attention soon focused on a possible link with colorectal cancer in humans. In 1988, Smith *et al* reported that mean fasting plasma gastrin was elevated eight-fold in patients with colon cancer and was also higher in those with adenomatous polyps compared to control patients (Smith *et al* 1988). Subsequently, a considerable number of studies have also addressed this question with several confirming the findings of Smith (Seitz *et al* 1989; Wong *et al* 1991; Charnley *et al* 1992; Seitz *et al* 1992). In contrast, a similar number have found no difference in circulating gastrin levels between colorectal tumour patients and controls (Suzuki *et al* 1988; Kauffmann and Ottenjann 1991; Creutzfeldt and Lamberts 1991; Yapp *et al* 1992; Kikendall *et al* 1992; Scotté *et al* 1992). The reasons for such divergent results are many and are discussed more fully later (Chapter 10) in relation to the study carried out in this thesis. In brief, several of the studies were not well designed with poor matching of patients and controls, failure to allow for possible effects of bowel preparation on gastrin levels, failure to control for

factors which may cause hypergastrinaemia and inappropriate statistical analyses. The majority of studies were conducted before the effects of *Helicobacter pylori* infection on gastrin concentrations became widely known and no investigation controlled for the presence of this common organism. To date, therefore, the question of whether patients with colorectal neoplasia have higher gastrin levels (for whatever reason) has not been adequately answered and there is a need for further rigorously controlled investigations. Data from a recent intriguing study has suggested that preoperative fasting gastrin concentrations correlated directly with the risk of liver metastases being present at the time of surgery (Kameyama *et al* 1993). The authors suggested that measurement of serum gastrin in combination with other parameters may be a useful predictor of liver metastases. After correcting for other factors, however, the patient numbers were small and it is unclear why the authors chose the cut-off value for gastrin that they did. The results may well have been non-significant if a higher or lower value had been chosen.

7.3 GASTRIN AS AN AUTOCRINE GROWTH FACTOR IN HUMAN CANCER

In the last decade or so it has become increasingly recognised that peptide hormones may function by mechanisms other than purely endocrine. Advances in tumour molecular biology have established the autocrine/paracrine hypothesis as being important in the control of cell proliferation (Sporn and Todaro 1980). Subsequently many hormones and growth factors have become recognised as relevant autocrine factors in common human malignancies (Cuttitta 1990).

Whether or not circulating gastrin is elevated in patients with colorectal tumours, investigators have recently examined the possibility that it may be an autocrine and/or paracrine trophic factor in this condition. In general, for a peptide to be accorded the status of an autocrine factor several criteria should ideally be fulfilled: tumour cells must produce the peptide with intact transcriptional, translational and post-translational processing pathways; peptide secretion should be demonstrated as should interaction of

the secreted peptide with membrane receptors; and growth stimulation of the same (or neighbouring cells) also ought to be seen.

An autocrine role for gastrin in colorectal cancer was first proposed by Hoosein *et al*, who found polyclonal antigastrin antibodies to inhibit the growth of two human cell lines *in vitro* when added to the medium (Hoosein *et al* 1989). Pre-incubation of the antiserum with human G-17 abrogated the effect. In a follow-up study, the authors confirmed this finding in three other cell lines, detected small amounts of a gastrin-like peptide in the cell culture supernatants by radioimmunoassay and found evidence for the presence of gastrin mRNA in the cells using northern analysis (Hoosein *et al* 1990). In contrast, Guo *et al* found the addition of antigastrin antibodies to MC-26 mouse colon cancer cells *in vitro* to have no effect on cell growth (Guo *et al* 1990).

Using a specific antigastrin antibody, with immunofluorescence and flow cytometry, Watson *et al* found six of twenty-eight freshly disaggregated human colorectal cancers to possess more than 20% gastrin-positive cells (Watson *et al* 1991). Tumour-free mucosa contained less than 5% gastrin-positive cells in the majority of cases. The authors suggested that foci of cells within the tumours may be able to secrete gastrin which may in turn stimulate proliferation of neighbouring gastrin receptor positive cells.

By northern analysis, no gastrin mRNA was found in two human cell lines but mRNA for the hormone was detected using the more sensitive polymerase chain reaction (PCR) technique (Baldwin *et al* 1990). The authors subsequently extended their findings and, using quantitative PCR, were able to detect gastrin mRNA in all seven colonic carcinoma cell lines tested with levels being two - seventy-fold greater than those of transforming growth factor-α (Baldwin and Zhang 1992). Singh's group in Texas have also recently described a PCR method for the detection of gastrin gene expression and found small but significant amounts of gastrin mRNA in the three human colon cancer cell lines tested (Xu *et al* 1994). Independently, others have found gastrin in samples of colon cancers but not in normal colonic mucosa (Monges *et al* 1993) and

demonstrated the capacity for gastrin transcription in some colon cancer cell lines (Tillotson *et al* 1993).

Following these reports of gastrin mRNA in colonic cancer cells, recent work has centred on synthesis of gastrin peptides in tumours. Using a panel of specific antibodies recognising different molecular forms of gastrin Kochman *et al* found equivalent amounts of progastrin in extracts of colorectal tumours and disease-free mucosa (Kochman *et al* 1992). While tumours contained more immature glycine-extended gastrins than normal mucosa, the latter contained greater amounts of mature, amidated gastrin. In comparison to gastric antrum the amounts detected were small and the ratio of amidated gastrin to glycine-extended gastrin was different, suggesting altered post-translational processing of gastrin in the tumours. No relationship between gastrin content and either tumour site or stage was seen. Around the same time, Dockray's group in Liverpool reported almost identical findings with all forty-four tumours examined containing progastrin and eleven having detectable mature gastrin (Nemeth *et al* 1993). Again mature gastrin was relatively more abundant in mucosa remote from the tumours, while the latter contained more progastrin. They confirmed these results with gel filtration and concluded that the gastrin gene is commonly expressed in colonic cancers but the ability to process the hormone fully is impaired. This was also the conclusion of Van Solinge *et al*, whose study also demonstrated the superior sensitivity of PCR methods over northern blot analysis for detecting small amounts of mRNA (Van Solinge *et al* 1993). While these authors felt the low level of expression of mature gastrin may not be relevant to tumour growth, preliminary results from others suggest that the more abundant non-amidated gastrin precursors may also possess trophic properties (Singh *et al* 1994). Several other groups have recently presented preliminary evidence that progastrin-derived glycine-extended intermediates may possess trophic properties, at least in AR42J cells (Seva *et al* 1994a; Nègre *et al* 1994). The possible importance of these precursors, traditionally regarded as lacking any biological activity, is discussed further in Chapter 12.

One problem with these studies is that they depend on tissue homogenisation for peptide extraction and nothing can therefore be said about the cellular source of the

gastrin. The use of extremely sensitive antibodies and PCR methods could conceivably be detecting small amounts of gastrin synthesis occurring in scattered neuroendocrine cells found in colonic crypts. One report suggested that *in situ* hybridisation may unfortunately not be sensitive enough to detect low level gastrin gene expression in colonic cancers (Xerri *et al* 1992). In a comprehensive study Finley *et al* have examined gastrin gene expression in the colon using immunohistochemistry, northern analysis and PCR (Finley *et al* 1993). In normal colonic mucosa immunohistochemistry revealed occasional crypt cells which stained for progastrin, gastrin and chromogranin A suggesting that gastrin is normally only expressed in these scattered cells. In contrast, twenty-two of twenty-three colon cancers had over 50% of cells staining for gastrin and progastrin and the majority of these cells were non-neuroendocrine as judged by the absence of chromogranin A staining. Interestingly, no gastrin staining was found in six benign polyps suggesting that gastrin synthesis is a late event in the carcinogenic process. The mRNA detected in the tumours by PCR was identical in sequence to that published for human gastrin. Very recently this group has confirmed these findings in two human colorectal cancer cell lines and found that somatostatin inhibited both gastrin mRNA expression and cell growth, the latter effect being prevented when gastrin was supplied along with somatostatin (Lebovitz *et al* 1993). Thus strong evidence exists that normal and malignant colonic epithelium may synthesise gastrin but the direct effects of this on cell proliferation and the biological importance of immature gastrin precursors on colorectal cancer cells remain to be shown beyond all doubt.

Evidence supporting a role for gastrin as an autocrine/paracrine growth factor in neoplasia outwith the colorectum has also been presented. Detailed discussion of these studies is outwith the scope of this thesis but such a role for gastrin has been proposed in gastric carcinoma cell lines (Van Solinge and Rehfeld 1992; Remy-Heintz *et al* 1993), a rat pancreatic carcinoma cell line (Blackmore and Hirst 1992), bronchogenic carcinomas (Rehfeld *et al* 1989), ovarian cancers (Van Solinge *et al* 1993), a human nephroblastoma cell line (Blackmore *et al* 1994) and a variety of uncommon neural and endocrine tumours (Rehfeld and Hilsted 1992). The ability to express and synthesise gastrin appears to be relatively common in many neoplastic cells

of diverse origins and may contribute to disordered growth control in these tumours. Future studies examining the effects of inhibition of gastrin gene expression on tumour cell growth will hopefully shed further light on the importance of gastrin as an autocrine/paracrine trophic factor.

CHAPTER 8

AIMS OF THESIS

8.1 INTRODUCTION

Much of the evidence presented above is consistent with a plausible role for gastrin as a biologically important trophic factor for normal and malignant colorectal epithelium. It is clear, however, that there are many conflicting studies yielding results which need to be clarified and also a number of questions which remain to be answered.

This is of direct clinical relevance for several reasons. Firstly, hypergastrinaemia occurs commonly in a variety of clinical situations, for example infection with *Helicobacter pylori*, pernicious anaemia and during treatment with acid-suppressing drugs such as H_2-antagonists and H^+/K^+-ATPase inhibitors. This last situation is important as these drugs are among the most widely prescribed worldwide and are being increasingly used for long term maintenance therapy of common acid-related disorders. Secondly, an increasing number of new, potent and highly specific gastrin/CCK-B receptor antagonists have been described and if gastrin is shown to be a biologically relevant trophic factor for colorectal neoplasia, then these drugs could be a useful additional therapeutic option in a disease which still carries an overall five-year survival rate of only 40-50 %.

8.2 PLAN OF INVESTIGATION

1. To study the effect of omeprazole-induced chronic endogenous hypergastrinaemia on chemical colorectal carcinogenesis in a well-established animal model.

Given the enormous number of prescriptions each year for acid-suppressing drugs, even a small enhancing effect of hypergastrinaemia on colorectal carcinogenesis would be important to detect. Despite the many studies of gastrin on animal models of colorectal cancer, none has investigated the possible role of chronic endogenous hypergastrinaemia resulting from prolonged treatment with such widely used drugs.

2. To clarify further the role of gastrin in human colorectal neoplasia.

Many aspects of this particular area remain unclear and to date no studies have addressed the issue *comprehensively* in one group of patients. This thesis aims to study circulating gastrin levels, tumour gastrin content and gastrin/CCK-B receptor content in the same group of patients and answer the following questions :-

a) Once all known factors causing hypergastrinaemia are controlled for, do fasting and meal-stimulated gastrin levels differ between tumour and control patients ?

b) In patients with colorectal cancer, do circulating gastrin concentrations fall following tumour resection, as has been suggested ?

c) Do tumours from these patients contain measurable amounts of gastrin and/or gastrin-processing intermediates and is there any correlation between tumour peptide content and circulating levels ?

d) Are measurable gastrin/CCK-B receptors present on the colorectal tumours of these patients and is there any correlation between circulating gastrin levels, tumour gastrin content and tumour gastrin receptor content ?

PART TWO

EXPERIMENTAL WORK

CHAPTER 9

OMEPRAZOLE AND EXPERIMENTAL COLONIC CARCINOGENESIS IN RATS

9.1 INTRODUCTION

To date very few studies have investigated the influence of drug-induced endogenous hypergastrinaemia on the development and/or progression of experimental colorectal tumours in animal models. Pawlikowski *et al* (1991) observed an increase in the number of vincristine-arrested metaphases in colonic crypts of rats treated with omeprazole compared to placebo. Others found omeprazole-induced hypergastrinaemia to have no effect on the growth rate of established gastrin-responsive murine (MC-26) colonic cancer xenografts (Graffner *et al* 1991).

The aim of this study was to assess the effects of omeprazole-induced hypergastrinaemia on the development of azoxymethane-induced colorectal carcinomas in rats. As described in Chapter 4 this model is well characterised and has become widely used in studies of factors influencing colorectal carcinogenesis.

9.2 MATERIALS AND METHODS

9.2.1 Experimental Animals

Five-week old female Sprague-Dawley rats (Harlan Olac Ltd., UK), weighing 125-150g were used throughout the study. Animals were maintained in the Joint Animal Facility of the University of Glasgow and housed two per cage, the cages being made of moulded polypropylene with stainless steel mesh lids. Animal bedding consisted of wood shavings and was changed weekly. Both experimental groups were kept in one room, separate from all other animals, under controlled environmental conditions (temperature 21°C ± 1°C, humidity 50% ± 5% and alternating 12 hour light-dark lighting cycle).

All rats were allowed food and water *ad libitum*. The diet consisted of standard rat and mouse formulation (Bantin and Kingman Ltd., Hull, UK). Animals were weighed once weekly until the end of carcinogen administration (week seventeen) and were then weighed three times per week. At each weighing, careful inspection was

made for signs of possible tumour development including inappetance, fur loss or textural change, abdominal swelling and rectal bleeding.

All procedures were licensed by the Home Office (Personal Investigator Licence No. PIL 60/03942) under the Animals (Scientific Procedures) Act 1986. In addition all work adhered to both the Code of Practice for the Joint Animal Facility, University of Glasgow and United Kingdom Coordinating Committee for Cancer Research (UKCCCR) guidelines.

9.2.2 Food Consumption

During weeks ten, fourteen, sixteen, twenty-one and twenty-four a measured quantity of food was provided for each cage and the amount remaining at the end of seven days weighed. This allowed calculation of the weight of diet consumed per cage per week and from this, the amount (gms.) eaten per rat per day was derived. Although this assumes that both rats in a given cage ate equal amounts, it was relative differences in food consumption between the two groups which were being sought, not absolute differences between individual rats and so this assumption is of less importance.

9.2.3 Experimental Design and Omeprazole Dosing

This is summarised diagrammatically in Figure 9.1. After a one week acclimatisation and quarantine period, rats were randomly divided into two groups to receive either omeprazole at a dose of $40\mu mol.kg^{-1}.day^{-1}$ or an equivalent volume of 0.25% buffered methylcellulose (the vehicle in which omeprazole was suspended), once daily by oral gavage. Gavage was carried out between 0900 and 1200 each day using a 2ml syringe attached to a stainless steel intragastric feeding tube (International Market Supplies, Cheshire, UK). Dosing was continued for the entire twenty-seven weeks of the study. Details of the method for reconstitution and formulation of omeprazole and dose calculation can be found in Appendices 2 and 3.

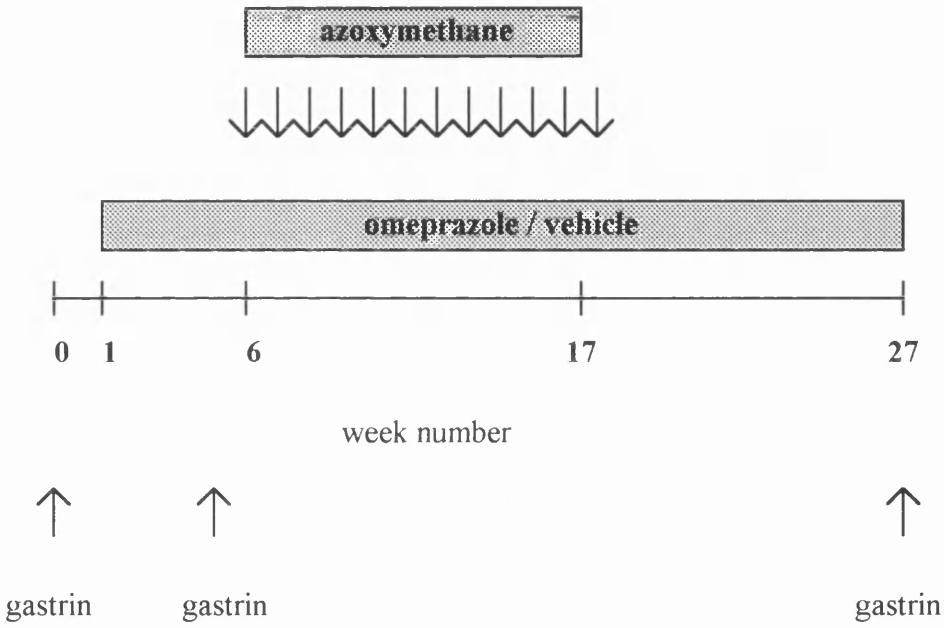


Figure 9.1. Schematic representation of study design.

9.2.4 Carcinogen Treatment

Azoxymethane (AOM) was purchased in 500mg ampoules from Sigma Chemical Co. (Poole, Dorset, UK), and stored at -20°C until required. The methods used for dilution, storage and handling azoxymethane (including safety precautions) are given in Appendix 1.

During weeks six to seventeen all rats received twelve weekly subcutaneous injections of azoxymethane at a dose of $10mg.kg.^{-1}$ $injection^{-1}$. Immediately prior to each injection of azoxymethane, rats were weighed and lightly anaesthetised with halothane. Based on the individual weight, the appropriate dose of AOM was then drawn up and injected into the paravertebral region. Injections were performed without knowledge of the group of origin of each rat as it was injected.

Because of the potent carcinogenic nature of AOM only authorised personnel wearing full protective clothing handled either the drug or recently injected animals. To minimise any potential exposure of personnel to exhaled carcinogenic metabolites (principally azomethane gas), all injections were performed between 1400 and 1600 on Friday afternoons. After each injection access to animals was denied for twenty-four hours except to authorised staff employing full protective measures. Furthermore, the animals were housed in a room with negative pressure ventilation to minimise any possible escape of exhaled carcinogenic metabolites.

9.2.5 Serum Gastrin Analysis

Serum gastrin levels in the two groups were measured at the following times:

(a) Week One - prior to commencing treatment with either omeprazole or vehicle.

Six freely-fed rats from each group were bled to measure baseline gastrin levels. Blood samples (0.7ml) were taken from the tail vein under light halothane anaesthesia.

(b) Week Five - prior to commencing azoxymethane injections.

In order to assess not only the peak gastrin levels obtained with omeprazole dosing, but also the duration of hypergastrinaemia over a twenty-four hour period between two doses, rats were bled at regular intervals over a twenty-four hour period. This was carried out immediately prior to dosing with either omeprazole or vehicle. As rat gastrin responses have been well documented elsewhere (Larsson *et al* 1986; Earl and Man 1990), only three rats from each group were selected at each time point. Blood (0.7ml) was again taken from the tail vein under halothane anaesthesia.

(c) Week Twenty-Seven - at termination of the experiment.

At death, blood was taken by cardiac puncture from all rats. Vehicle-treated rats were bled four hours after the last dose whereas omeprazole-treated rats were bled either four hours (n=6) or twenty-four hours (n=13) after the final dose of the drug.

Blood samples were collected on ice in plain tubes, allowed to clot and centrifuged at 3000 r.p.m. for ten minutes at 4°C to separate serum. Serum was then stored at -70°C until analysis which was performed (in duplicate) by radioimmunoassay using antibody R98 (Ardill 1973). This recognises the C-terminal end of both human and rat gastrin and has a sensitivity of approximately $3ngL^{-1}$.

9.2.6 Post mortem analysis

At the end of the study in week twenty-seven, animals were killed by exsanguination under CO_2 anaesthesia. All animals were killed between 1200 and 1600 to minimise possible effects of diurnal variation on serum gastrin levels. Following division of the pubic symphisis the entire colon from anorectal junction to caecum was excised, opened along the length of the antimesenteric border, pinned flat on a cork mat with the mucosal surface uppermost and rinsed thoroughly with ice-cold 0.9% saline to remove faeces and clot. Total colon length was measured and the number, position (in

millimetres from the anorectal junction) and volumes (the product of length x width x depth in mm^3) of all lesions recorded. Samples of liver (approximately 1g) and macroscopically normal ascending and descending taken from 75% and 25% along the length of the colon, respectively, were snap frozen in liquid nitrogen and stored at -70°C until further analysis.

After the remainder of the colon and liver had been fixed overnight in 10% neutral-buffered formalin, all lesions plus samples of macroscopically ascending and descending colon (as above) and liver were removed, individually coded, processed for routine histopathology and embedded in paraffin wax. Sections (4μm) were stained with haematoxylin and eosin and examined "blind" i.e. without knowledge of their group of origin. As previously described (Sunter *et al* 1978), all neoplastic lesions were described as either benign adenomas or adenocarcinomas, the latter being classified into Group 1-3 carcinomas according to the degree of invasiveness and differentiation.

9.2.7 Chemicals

Pure omeprazole (a gift from Dr. H. Mattsson, Astra Hässle, AB Mölndal, Sweden) was suspended in 0.25% methylcellulose (Dow Chemicals), buffered with $2mg.ml^{-1}$ sodium bicarbonate and adjusted to pH 9.0 with sodium hydroxide. The solution was stored at -20°C prior to use and fresh aliquots thawed for use every four to five days and kept at 4°C. Azoxymethane (Sigma Chemical Co., Dorset, UK) was dissolved in sterile 0.9% sodium chloride, kept as a stock solution of $100mg.ml^{-1}$ at 4°C and further diluted to a final concentration of $10mg.ml^{-1}$ with sodium chloride immediately prior to use. Further details are given in Appendix 3.

9.2.8 Statistics

All statistical testing was performed using the statistical software package MINITAB 8.0 (Minitab Inc., USA). Tumour incidence was analysed by Fischer's exact test and tumour distribution and multiplicity by the Mann Whitney U-Test. Correlations between animal weight and tumour development were assessed using Spearman's rank

correlation coefficient. The unpaired t-test was used for all other tests including tumour volumes after logarithmic transformation of the data. Statistical significance was taken as a value of $P < 0.05$ in all tests.

9.3 RESULTS

9.3.1 Animal Survival

One animal in the omeprazole group (rat number eight) died during week eleven as a result of oesophageal perforation during oral gavage. At necropsy a pneumothorax and mediastinitis were found but close examination of the opened colon and rectum revealed no evidence of tumour formation or other abnormality. A further rat (number sixteen) in the omeprazole group developed respiratory distress and weight loss of greater than ten per cent and was therefore killed in week twenty-two. Again, no abnormality was found in the large intestine or elsewhere in either the small intestine, peritoneal cavity or liver at necropsy. There was evidence, however, of pulmonary congestion and significant frothy upper airways secretions, suggesting either pulmonary aspiration or oesophageal perforation during oral gavage.

In the vehicle-treated group two rats (numbers one and ten) developed diarrhoea and rectal bleeding in week twenty-five. At necropsy both were found to have colonic tumours. All other animals survived until the end of the study and were killed in week twenty-seven.

9.3.2 Animal Growth and Development

Both groups of animals grew at similar rates although the omeprazole-treated group were consistently lighter throughout the study (Figure 9.2). At the end of the experiment the omeprazole-treated rats weighed (± S.D.) 342.5 (± 41.7) g compared to a mean weight of 389.3 (± 44.6) g in vehicle-treated control animals ($P = 0.004$). Throughout the study period all animals in both groups appeared healthy and no differences in fur quality or other subjective measures of animal wellbeing were noted

Rat No.	O 1	V 1	O 2	V 2	O 3	V 3	O 4	V 4	O 5	V 5
1	176	174	212	226	229	235	252	262	264	271
2	162	177	233	270	250	300	266	320	258	340
3	163	156	232	208	254	224	272	246	280	260
4	156	196	204	268	224	283	240	307	260	323
5	188	190	229	243	253	282	266	286	279	302
6	156	156	225	207	234	224	253	242	254	250
7	185	169	227	215	244	226	269	243	252	254
8	176	169	214	225	227	236	253	254	285	267
9	180	170	229	244	253	271	268	297	258	317
10	158	172	211	246	227	266	261	292	265	315
11	167	171	218	233	226	246	252	266	227	282
12	179	154	243	199	251	218	288	235	279	244
13	150	174	222	231	224	246	254	269	219	278
14	161	180	233	246	235	262	270	284	265	291
15	162	160	191	241	200	260	226	293	226	310
16	181	172	239	228	255	242	270	286	273	307
17	164	185	190	252	202	261	218	281	240	307
18	170	166	209	244	230	233	265	250	239	251
19	171	174	201	244	213	259	220	282	249	290
20	176	162	226	214	246	243	264	261	251	279
Mean	**169.05**	**171.35**	**219.40**	**234.20**	**233.85**	**250.85**	**256.35**	**272.80**	**256.15**	**286.90**
S.D.	**10.71**	**11.00**	**15.05**	**19.30**	**16.82**	**22.42**	**18.21**	**23.57**	**18.87**	**27.65**

Table 9.1. (5 pages). Weekly animal weights (gms) during experimental period. O = omeprazole, V = vehicle.

Rat No.	O 6	V 6	O 7	V 7	O 8	V 8	O 9	V 9	O 10	V 10
1	265	272	276	272	277	286	285	277	286	280
2	268	352	276	348	270	336	273	330	274	330
3	276	269	286	274	288	271	266	276	284	277
4	262	336	268	334	275	350	280	329	287	325
5	284	311	294	312	299	306	267	296	280	308
6	258	254	264	264	267	263	299	263	310	270
7	260	273	272	265	276	276	326	273	331	280
8	291	280	317	290	320	290	282	289	289	296
9	264	328	276	332	280	331	286	318	296	329
10	272	330	282	343	284	342	280	334	292	346
11	230	292	230	300	238	302	238	262	244	304
12	288	248	290	252	298	259	302	302	300	267
13	222	282	230	298	238	303	242	310	247	311
14	274	303	284	305	292	305	292	310	306	317
15	224	318	232	313	237	319	286	292	250	296
16	266	319	277	323	280	325	244	310	295	309
17	244	306	271	306	270	321	270	336	281	354
18	243	266	268	274	262	274	274	320	274	321
19	253	301	265	312	267	312	268	282	280	289
20	251	281	272	286	278	295	280	308	292	318
Mean	**259.75**	**296.05**	**271.50**	**300.15**	**274.80**	**303.30**	**277.00**	**300.85**	**284.90**	**306.35**
S.D.	**19.71**	**28.86**	**21.29**	**27.76**	**20.85**	**26.57**	**20.80**	**23.44**	**21.03**	**24.49**

Table 9.1 contd. (page 2 of 5).

Rat No.	O 11	V 11	O 12	V 12	O 13	V 13	O 14	V 14	O 15	V 15
1	286	284	294	284	295	289	296	290	301	294
2	276	348	287	350	292	367	287	369	294	364
3	288	279	290	282	291	288	304	288	302	295
4	294	348	295	348	291	346	294	357	299	295
5	281	313	287	309	295	314	299	324	298	351
6	314	275	314	274	314	279	322	283	325	335
7	291	282	295	289	302	286	303	287	310	285
8	291	294	*	294	*	300	*	296	*	299
9	307	337	306	328	310	318	317	326	315	338
10	294	360	292	355	294	359	299	359	302	369
11	244	308	254	313	253	315	258	310	251	324
12	303	271	318	272	310	280	310	282	318	280
13	254	316	267	320	265	320	265	318	271	328
14	308	318	314	320	312	327	319	321	332	330
15	255	296	258	299	259	302	264	300	264	312
16	298	303	313	314	316	320	316	314	323	314
17	284	344	285	351	284	355	294	351	299	363
18	298	314	299	319	312	328	315	328	312	339
19	289	290	288	294	294	303	297	304	297	301
20	295	328	298	328	308	340	308	341	316	346
Mean	**287.50**	**310.40**	**292.32**	**312.15**	**294.58**	**316.80**	**298.26**	**317.40**	**301.53**	**323.10**
S.D.	**18.34**	**26.94**	**17.84**	**26.04**	**18.51**	**26.60**	**18.71**	**27.02**	**20.79**	**27.50**

Table 9.1 contd. (page 3 of 5).

Rat No.	O 16	V 16	O 17	V 17	O 18	V 18	O 19	V 19	O 20	V 20	O 21	V 21
1	304	296	308	301	316	304	318	310	317	326	311	329
2	301	375	301	380	298	394	302	405	304	424	279	417
3	303	300	311	303	314	308	321	317	314	322	303	331
4	302	372	305	376	312	386	321	393	322	391	306	393
5	302	334	304	339	302	340	305	351	311	358	319	357
6	333	288	336	289	340	294	352	301	347	299	354	304
7	316	300	320	308	324	302	316	309	333	318	337	320
8	*	309	*	317	*	314	*	326	*	324	*	330
9	326	339	322	345	332	348	328	352	328	350	337	358
10	302	381	308	396	320	410	321	418	321	432	336	438
11	266	329	267	339	264	334	272	340	274	344	286	344
12	320	287	318	294	324	298	326	296	322	299	328	301
13	278	336	281	345	286	348	270	348	270	362	277	362
14	321	337	326	346	330	352	340	355	344	370	352	374
15	267	315	265	319	268	324	288	326	290	320	292	333
16	340	329	336	331	338	330	338	342	342	359	358	363
17	300	369	304	378	308	398	321	398	319	399	290	410
18	326	349	325	354	328	364	334	379	335	390	302	396
19	310	312	301	314	324	316	316	325	318	332	319	330
20	324	347	329	348	336	346	336	354	339	375	343	380
Mean	**307.42**	**330.20**	**308.79**	**336.10**	**313.89**	**340.50**	**317.11**	**347.25**	**318.42**	**354.70**	**317.32**	**358.50**
S.D.	**20.46**	**29.29**	**20.35**	**30.67**	**21.97**	**35.14**	**21.80**	**35.67**	**21.70**	**38.58**	**26.01**	**38.11**

Table 9.1 contd. (page 4 of 5).

Rat No.	O 22	V 22	O 23	V 23	O 24	V 24	O 25	V 25	O 26	V 26	O 27	V 27
1	324	339	342	335	336	347	333	335	331	*	329	*
2	303	434	312	444	320	467	323	478	320	481	323	497
3	333	344	324	339	333	347	337	346	340	356	357	352
4	334	398	339	401	346	417	348	414	348	419	345	420
5	329	370	330	372	335	390	336	383	349	385	n/a	385
6	358	312	367	310	386	326	388	323	387	320	390	n/a
7	344	331	340	326	340	341	342	333	339	337	315	n/a
8	*	339	*	344	*	350	*	349	*	354	*	330
9	340	381	345	383	348	401	348	395	348	406	440	407
10	335	465	331	460	334	484	334	470	328	*	393	*
11	291	353	289	347	298	361	296	358	298	358	299	358
12	327	305	325	304	329	322	326	318	332	320	334	324
13	272	367	270	366	278	383	280	378	286	376	269	376
14	354	387	352	380	359	399	364	390	366	392	352	392
15	296	353	298	349	306	370	307	367	314	372	294	375
16	366	375	365	372	370	390	374	388	372	397	*	416
17	*	413	*	414	*	430	*	424	*	427	354	418
18	344	406	350	406	364	426	364	410	364	409	374	426
19	325	332	326	334	333	342	332	343	336	339	310	339
20	347	403	345	402	360	418	364	417	365	415	345	419
Mean	**329.00**	**370.35**	**330.56**	**369.40**	**338.00**	**385.50**	**338.67**	**381.00**	**340.17**	**381.28**	**342.5**	**389.3**
S.D.	**24.63**	**41.24**	**25.38**	**42.36**	**27.07**	**45.30**	**27.19**	**45.30**	**25.87**	**41.76**	**41.7**	**44.6**

Table 9.1 contd. (page 5 of 5).

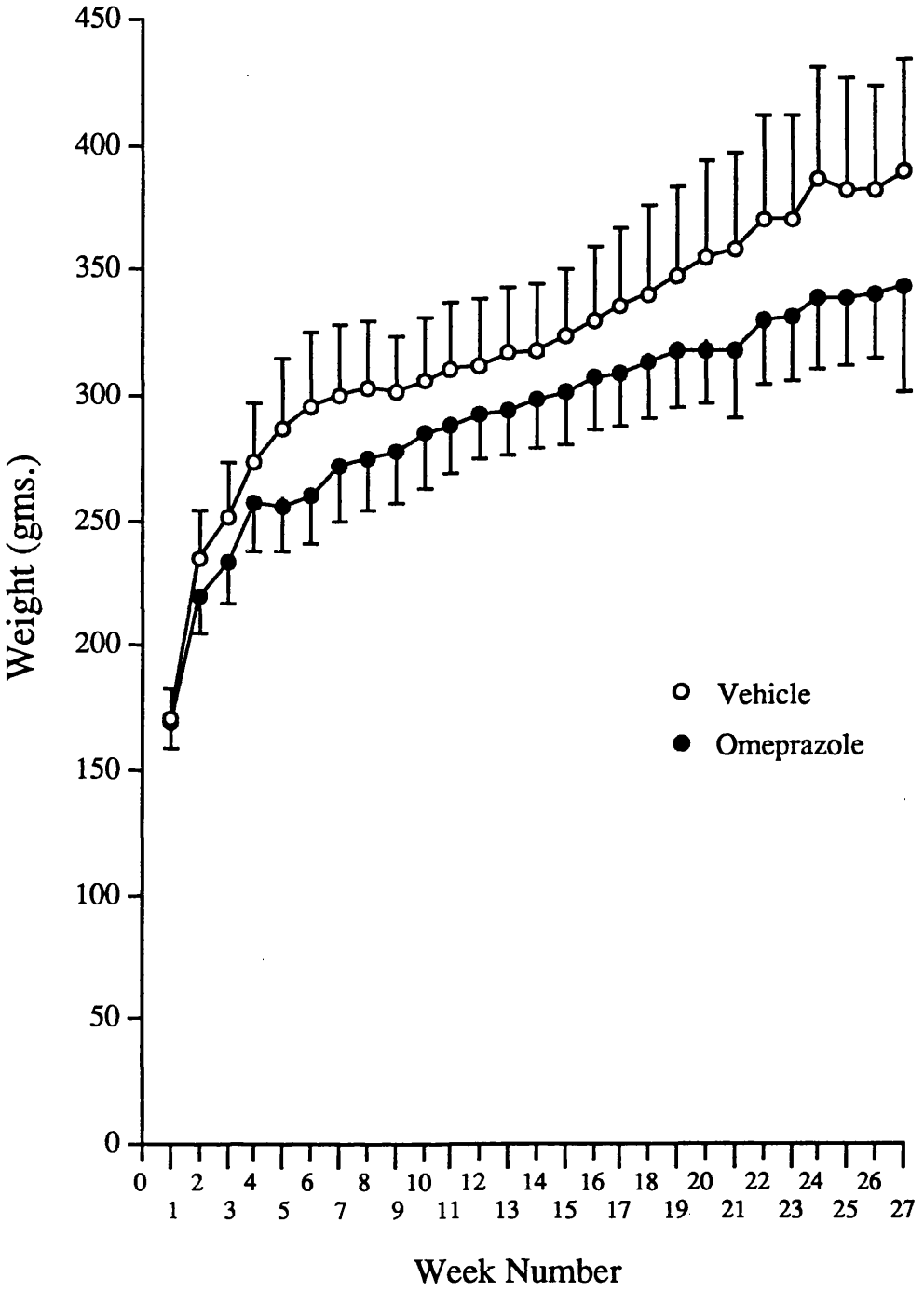


Figure 9.2

Rat growth during study period (mean ± S.D.)

between the groups. Individual animal weights are detailed in Table 9.1. All animals, except the omeprazole-treated rat which died during week eleven were included in effective numbers for analysis.

9.3.3 Food Consumption

No consistent pattern in differences in food consumption was found throughout the study (Tables 9.2 and 9.3). Calculated food consumption, expressed as grams per rat per day, was significantly greater in the omeprazole group in week ten ($P < 0.05$) and week fourteen ($P < 0.001$). In contrast, food intake was higher in vehicle-treated animals in week twenty-one ($P < 0.001$) whereas no significant difference was noted in either of the two other weeks when food consumption was measured (weeks sixteen and twenty-four).

9.3.4 Serum Gastrin Levels

(a) Week One - Six freely-fed rats from each group were bled in week one for measurement of baseline serum gastrin concentrations. Mean gastrin levels (± S.D.) in the omeprazole group were 204 (± 57) $ng.L^{-1}$ compared to 220 (± 61) $ng.L^{-1}$ in the vehicle group (P = not significant). Results are shown in Figure 9.3 and Table 9.4 shows individual gastrin concentrations in the two groups of rats.

(b) Week Five - Omeprazole treatment resulted in greatly elevated serum gastrin concentrations with peak levels occurring two hours after dosing. Peak values in the omeprazole group were 1700 $ng.L^{-1}$ (an approximate nine-fold increase) and levels remained elevated for most of the twenty-four hour period. Even twenty hours after dosing (at 0800) mean gastrin concentration ($383ngL^{-1}$) was still twice that in the vehicle group ($184ngL^{-1}$). In contrast, little variation in serum gastrin concentrations occurred throughout the twenty-four hour period following dosing with vehicle. The results are depicted in Figure 9.4 and individual gastrin levels are shown in Table 9.5.

	week 10	week 14	week 16	week 21	week 24
Omeprazole	18.24 ± 0.92 *	19.76 ± 1.43 **	19.02 ± 1.39	19.75 ± 0.98	19.08 ± 2.30
Vehicle	17.15 ± 1.17	16.61 ± 0.83	19.90 ± 0.75	22.79 ± 1.56 **	18.73 ± 2.45

Table 9.2. Mean (± SD) food consumption.
Results are expressed as gms.rat^{-1}.day^{-1}. * $P < 0.05$. ** $P < 0.001$.

	week 10	week 14	week 16	week 21	week 24
cage 1	17.30	18.40	20.40	18.50	20.30
cage 2	18.40	20.00	20.40	19.90	21.40
cage 3	19.00	19.90	17.10	19.00	20.60
cage 4	19.20	18.10	18.40	20.10	15.40
cage 5	18.10	20.90	19.90	19.80	20.30
cage 6	16.60	18.10	19.60	19.10	16.90
cage 7	18.70	19.00	17.90	18.70	17.60
cage 8	17.30	20.10	17.10	19.90	16.40
cage 9	19.40	22.70	18.60	21.60	22.00
cage 10	18.40	20.40	20.80	20.90	19.90
Mean	**18.24**	**19.76**	**19.02**	**19.75**	**19.08**
S.D.	**0.92**	**1.43**	**1.39**	**0.98**	**2.30**

Table 9.3a. Food consumption per cage ($gms.rat^{-1}.day^{-1}$) for Omeprazole group.

	week 10	week 14	week 16	week 21	week 24
cage 1	16.60	18.20	20.00	24.20	21.10
cage 2	14.70	15.90	19.40	21.60	15.70
cage 3	17.50	15.90	19.90	22.90	17.10
cage 4	16.30	15.60	19.50	21.30	15.60
cage 5	18.30	17.30	21.40	25.70	22.80
cage 6	16.70	16.60	19.30	20.50	21.60
cage 7	17.80	16.10	20.10	21.70	18.90
cage 8	17.00	16.60	19.00	23.90	18.60
cage 9	17.80	16.40	19.50	22.90	18.50
cage 10	18.80	17.50	20.90	23.20	17.40
Mean	**17.15**	**16.61**	**19.90**	**22.79**	**18.73**
S.D.	**1.17**	**0.83**	**0.75**	**1.56**	**2.45**

Table 9.3b. Food consumption per cage ($gms.rat^{-1}.day^{-1}$) for Vehicle group.

Rat Number	Omeprazole	Vehicle
1	225	180
2	165	120
3	300	220
4	195	260
5	135	260
6	195	280
Mean	**204**	**220**
S.D.	**57**	**61**

Table 9.4. Serum gastrin concentrations ($ng.L^{-1}$) in six rats per group in week one.

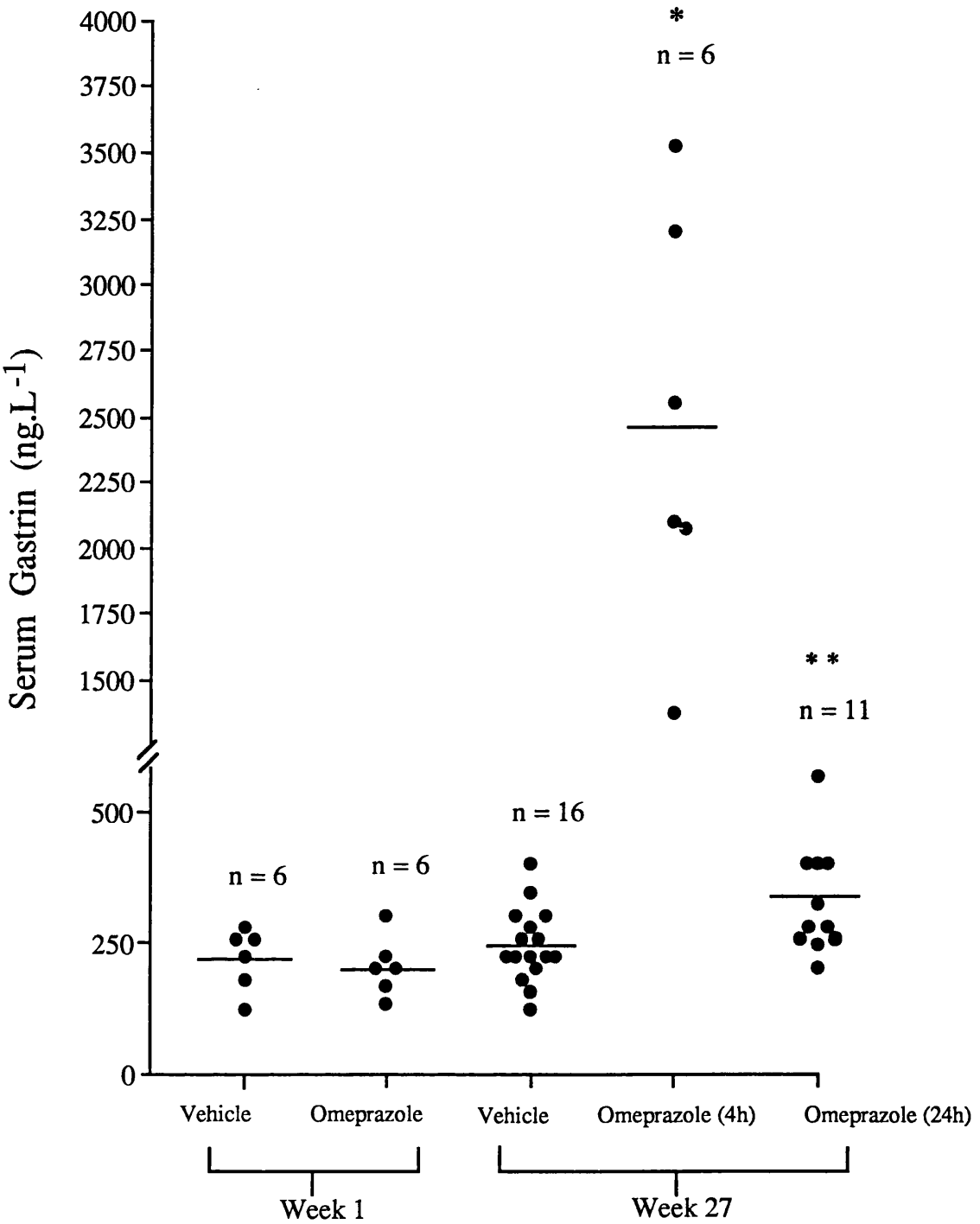


Figure 9.3

Serum gastrin levels during week 1 and at death (week 27). Omeprazole-treated rats were killed either 4 or 24 hours after the last dose.
Horizontal bars indicate mean values.
*P = 0.001. **P < 0.05.

Time

Rat Number	1200	1400	1600	2000	2400	0400	0800	1200
Omeprazole 1	250	1000	-	2000	1400	-	700	200
Omeprazole 2	400	600	-	750	1600	-	475	450
Omeprazole 3	350	3600	-	1200	1400	-	350	200
Mean	**333**	**1733**	-	**1316**	**1467**	-	**508**	**283**
Vehicle 1	190	-	225	190	405	360	150	-
Vehicle 2	180	-	165	135	195	255	165	-
Vehicle 3	165	-	300	195	95	135	255	-
Mean	**178**	-	**230**	**173**	**230**	**250**	**190**	-

Table 9.5. Mean serum gastrin concentrations ($ng.L^{-1}$) over a 24-hour period (week five).

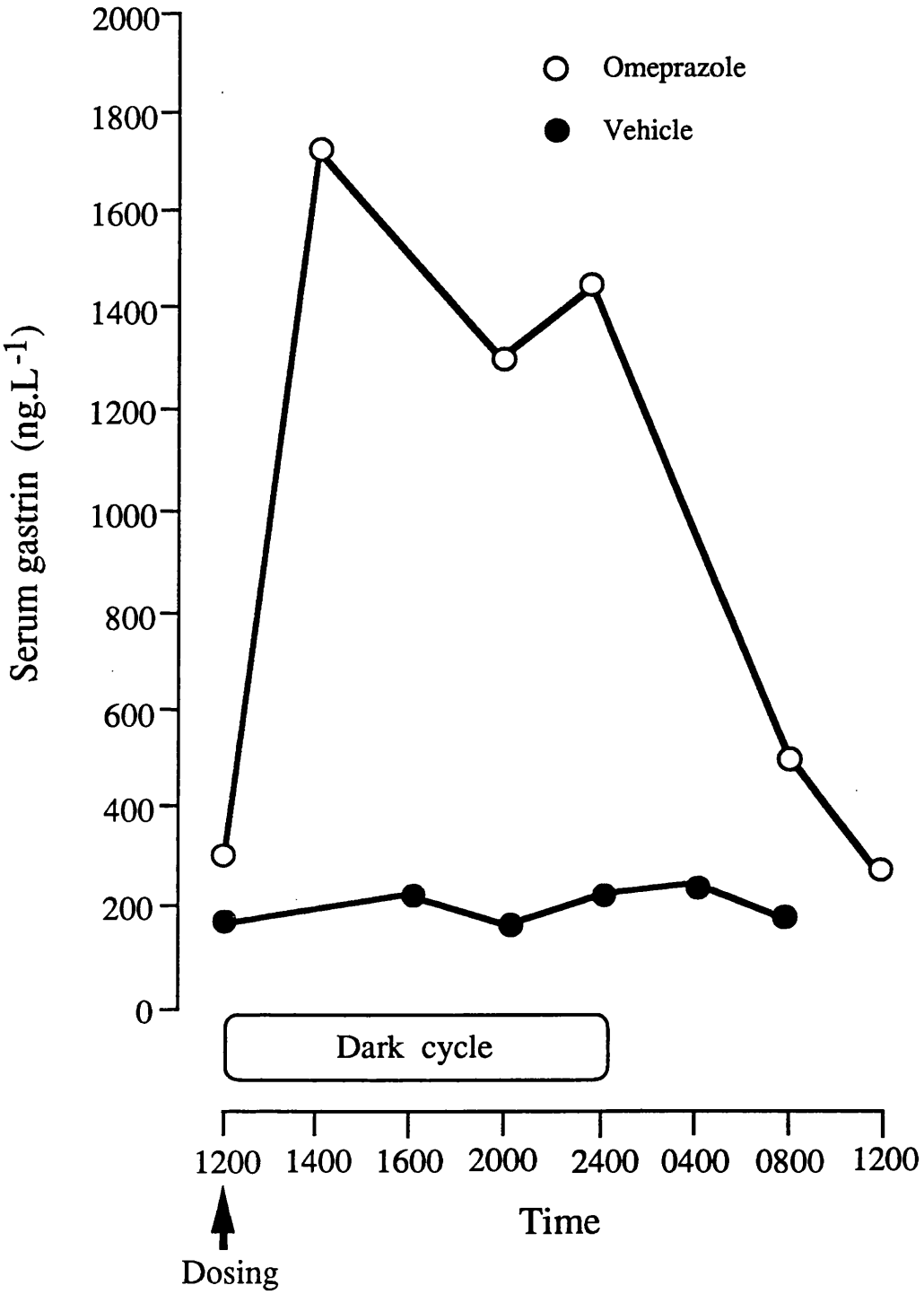


Figure 9.4

Serum gastrin concentrations ($ng.L^{-1}$) during a 24 hour period in week 5.

(c) Week Twenty-Seven - Two samples taken from omeprazole-treated rats twenty-four hours after the final dose of omeprazole were haemolysed and unsuitable for analysis. Mean serum gastrin values in omeprazole-treated rats both four hours (2470 ± 796 $ng.L^{-1}$, $P = 0.001$) and twenty-four hours (331 ± 113ngL^{-1}, $P < 0.05$) after the last dose were significantly higher than in vehicle-treated controls (245 ± 72ngL^{-1}). The results are represented in Figure 9.3 and Table 9.6. Figure 9.3 also shows the considerable variation in gastrin levels in response to omeprazole with levels four hours after dosing ranging from 1360-3520 $ng.L^{-1}$. There was also a three-fold variation among rats in the gastrin levels twenty-four hours after the last dose (range 200-600 $ng.L^{-1}$).

9.3.5 Tumour Development

(i) Tumour Incidence and Number

Significantly fewer rats in the omeprazole-treated group (12/19, 63%) developed histologically confirmed tumours, compared to the rats treated with vehicle only (19/20, 95%; $P < 0.02$, Fischer's exact test). Furthermore, in the omeprazole group both the total number of tumours (twenty-eight) and the median number of tumours per rat (1.0, range 0-5) were significantly lower ($P = 0.02$, Mann-Whitney U test) than in the vehicle-treated group (fifty-nine tumours, median 3.0, range 0-10). Close examination of sections taken from samples of macroscopically normal ascending and descending colon did not reveal any early or microscopic lesions. Liver metastases were not found in either group but deposits of tumour were seen in the omentum of one rat in the vehicle-treated group. The results are shown in Table 9.7.

(ii) Tumour Histology

Histologically the relative numbers of benign adenomas and adenocarcinomas were similar in both groups with six adenomas (21%) and twenty-two carcinomas (79%) in the omeprazole-treated rats compared to eleven adenomas (19%) and forty-eight (81%)

Rat Number	Omeprazole (4 hours)	Omeprazole (24 hours)	Vehicle (4 hours)
1	1360	-	n/a
2	2080	-	340
3	2560	-	180
4	3520	-	n/a
5	3200	-	280
6	2100	-	220
7	-	600	260
8	-	*	260
9	-	320	120
10	-	400	220
11	-	280	n/a
12	-	200	200
13	-	240	220
14	-	280	300
15	-	400	300
16	-	260	400
17	-	260	220
18	-	400	220
19	-	n/a	160
20	-	n/a	n/a
Mean	**2470**	**331**	**245**
S.D.	**796**	**113**	**72**

Table 9.6. Individual serum gastrin concentrations ($ng.L^{-1}$) at death (week twenty-seven). *Rat excluded. n/a = no result.

Group	No. Rats	Tumour +ve rats	Tumours/ rat †	Total tumours/ group	Adenomas	Adeno-carcinomas	Rats with metastasis
Omeprazole	19	12 *	1.0 (0-5) **	28 **	6	22	0
Vehicle	20	19	3.0 (0-10)	59	11	48	1

Table 9.7. Tumour development. * $P < 0.02$. ** $P = 0.02$. †median (range).

carcinomas in rats treated with vehicle. In the vehicle-treated group, three tumours had typical cytological features of malignancy but invasiveness could not be assessed because the tumour stalk was not adequately seen and these lesions have been termed "carcinomas unclassified". The relative proportions of Group 1 and Group 2 carcinomas were similar in the two groups of animals but there were considerably more Group 3 carcinomas (nine, 15%) in vehicle-treated animals than in the omeprazole-treated group (one, 3.6%). Tumour classification is detailed in Tables 9.8 - 9.10. Examples of azoxymethane-induced tumours are shown in Figures 9.5 - 9.7.

(iii) Tumour Distribution

There was no significant difference between the groups in the site distribution of the tumours, the majority occurring in the distal colon and rectum in each group. Mean colon length was 196 (± 14.7) mm in the Omeprazole group and 201 (± 16.8) mm in the Vehicle group ($P = 0.35$). To control for variations in colon length among animals, tumour sites were expressed as percentage distance along the length of the colon with '0' representing the anorectal margin and '100' representing the caecum. In both groups the majority of lesions occurred in the distal colon and rectum, the median distances being 32.5% (range 3-95%) for the Omeprazole group and 31% (range 3-70%) for the Vehicle group ($P = 0.86$, Mann-Whitney U test). Tumour site distributions are detailed in Table 9.11 and 9.12 and graphically represented in Figure 9.8.

(iv) Tumour Volumes

There was no significant difference in average tumour size in the two groups as measured by the median tumour volume, the results being 27mm^3 (range 1-288mm^3) for the Omeprazole group and 30mm^3 (range 1-800mm^3) for the Vehicle group ($P = 0.90$, Mann-Whitney U test). There was, however, a preponderance of small tumours (< 10mm^3) in the vehicle group (22/59, 37%) compared to the omeprazole group (4/28, 14%). Table 9.13 and 9.14 and Figure 9.9 show the tumour volume results.

Group	No. Rats	Total no. tumours	Benign adenomas	Group 1 carcinomas	Group 2 carcinomas	Group 3 carcinomas	Unclassified carcinomas
Omeprazole	19	28	6	5	16	1	0
Vehicle	20	59	11	7	29	9	3

Table 9.8. Histological classification of tumours (see text).

Animal number	Omeprazole	Vehicle
1	0	10
2	2	3
3	1	3
4	4	2
5	0	5
6	2	3
7	4	4
8	*	3
9	1	3
10	1	4
11	0	1
12	0	0
13	2	1
14	0	1
15	5	2
16	0	1
17	0	4
18	1	5
19	4	2
20	1	2
Total	**28**	**59**
Median	**1.0**	**3.0**
Range	**0 - 5**	**0 - 10**

Table 9.9. Number of histologically confirmed tumours per rat. * Rat excluded.

Rat No.	No. of Lesions	Lesion No.	Histological Classification
1	0		
2	2	1	benign adenoma
		2	group 3 carcinoma
3	1	1	group 2 carcinoma
4	4	1	group 1 carcinoma
		2	group 2 carcinoma
		3	group 2 carcinoma
		4	group 2 carcinoma
5	0		
6	2	1	group 2 carcinoma
		2	group 1 carcinoma
7	4	1	group 2 carcinoma
		2	group 2 carcinoma
		3	benign adenoma
		4	benign adenoma
8	*	*	*
9	1	1	group 2 carcinoma
10	1	1	group 1 carcinoma

Table 9.10a. (2 pages). Histological classification of tumours in Omeprazole group.

Rat No.	No. of Lesions	Lesion No.	Histological Classification
11	0		
12	0		
13	2	1	benign adenoma
		2	group 2 carcinoma
14	0		
15	5	1	group 1 carcinoma
		2	group 2 carcinoma
		3	group 2 carcinoma
		4	benign adenoma
		5	benign adenoma
16	0		
17	0		
18	1	1	group 2 carcinoma
19	4	1	group 2 carcinoma
		2	group 2 carcinoma
		3	group 2 carcinoma
		4	group 1 carcinoma
20	1	1	group 2 carcinoma

Table 9.10a contd.(page 2 of 2). Histological classification of tumours in Omeprazole group.

Rat No.	No. of Lesions	Lesion No.	Histological Classification
1	10	1	group 1 carcinoma
		2	group 2 carcinoma
		3	benign adenoma
		4	group 2 carcinoma
		5	group 2 carcinoma
		6	group 2 carcinoma
		7	group 2 carcinoma
		8	group 2 carcinoma
		9	benign adenoma
		10	carcinoma - unclassified
2	3	1	group 2 carcinoma
		2	group 2 carcinoma
		3	group 3 carcinoma
3	3	1	group 2 carcinoma
		2	group 2 carcinoma
		3	group 3 carcinoma
4	2	1	benign adenoma
		2	benign adenoma
5	5	1	group 3 carcinoma
		2	group 2 carcinoma
		3	group 1 carcinoma
		4	group 2 carcinoma
		5	group 2 carcinoma

Table 9.10b (3 pages). Histological classification of tumours in Vehicle group.

Rat No.	No. of Lesions	Lesion No.	Histological Classification
6	3	1	group 2 carcinoma
		2	group 1 carcinoma
		3	group 2 carcinoma
7	4	1	benign adenoma
		2	group 2 carcinoma
		3	carcinoma - unclassified
		4	carcinoma - unclassified
8	3	1	group 1 carcinoma
		2	group 2 carcinoma
		3	benign adenoma
9	3	1	group 1 carcinoma
		2	group3 carcinoma
		3	group 2 carcinoma
10	4	1	group 2 carcinoma
		2	group 1 carcinoma
		3	group3 carcinoma
		4	benign adenoma
11	1	1	group 3 carcinoma
12	0		

Table 9.10b contd. (page 2 of 3). Histological classification of tumours in Vehicle group.

Rat No.	No. of Lesions	Lesion No.	Histological Classification
13	1	1	group 2 carcinoma
14	1	1	group 2 carcinoma
15	2	1	benign adenoma
		2	group 2 carcinoma
16	1	1	group 2 carcinoma
17	4	1	benign adenoma
		2	group 2 carcinoma
		3	group 2 carcinoma
		4	group 3 carcinoma
18	5	1	benign adenoma
		2	benign adenoma
		3	group 2 carcinoma
		4	group 2 carcinoma
		5	group 3 carcinoma
19	2	1	group 3 carcinoma
		2	group 2 carcinoma
20	2	1	group 2 carcinoma
		2	group 1 carcinoma

Table 9.10b contd. (page 3 of 3). Histological classification of tumours in Vehicle group.

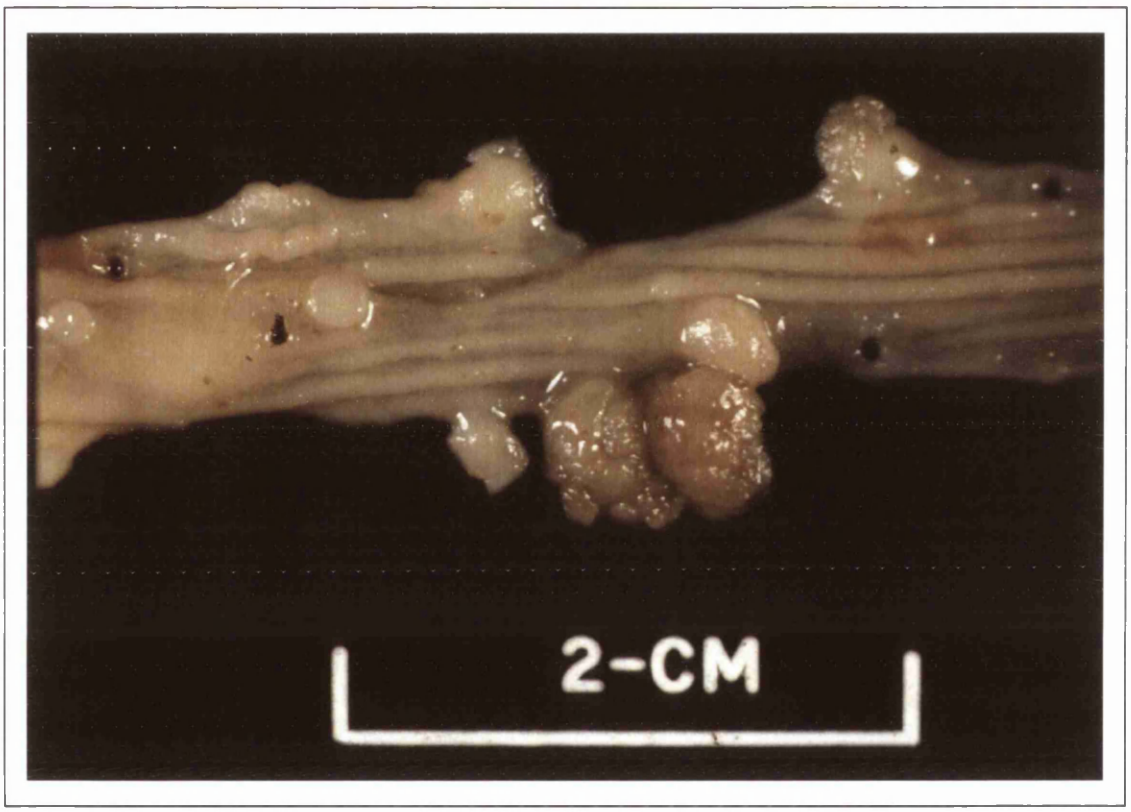


Figure 9.5
Multiple tumours of varying stages induced by azoxymethane in a vehicle-treated rat.

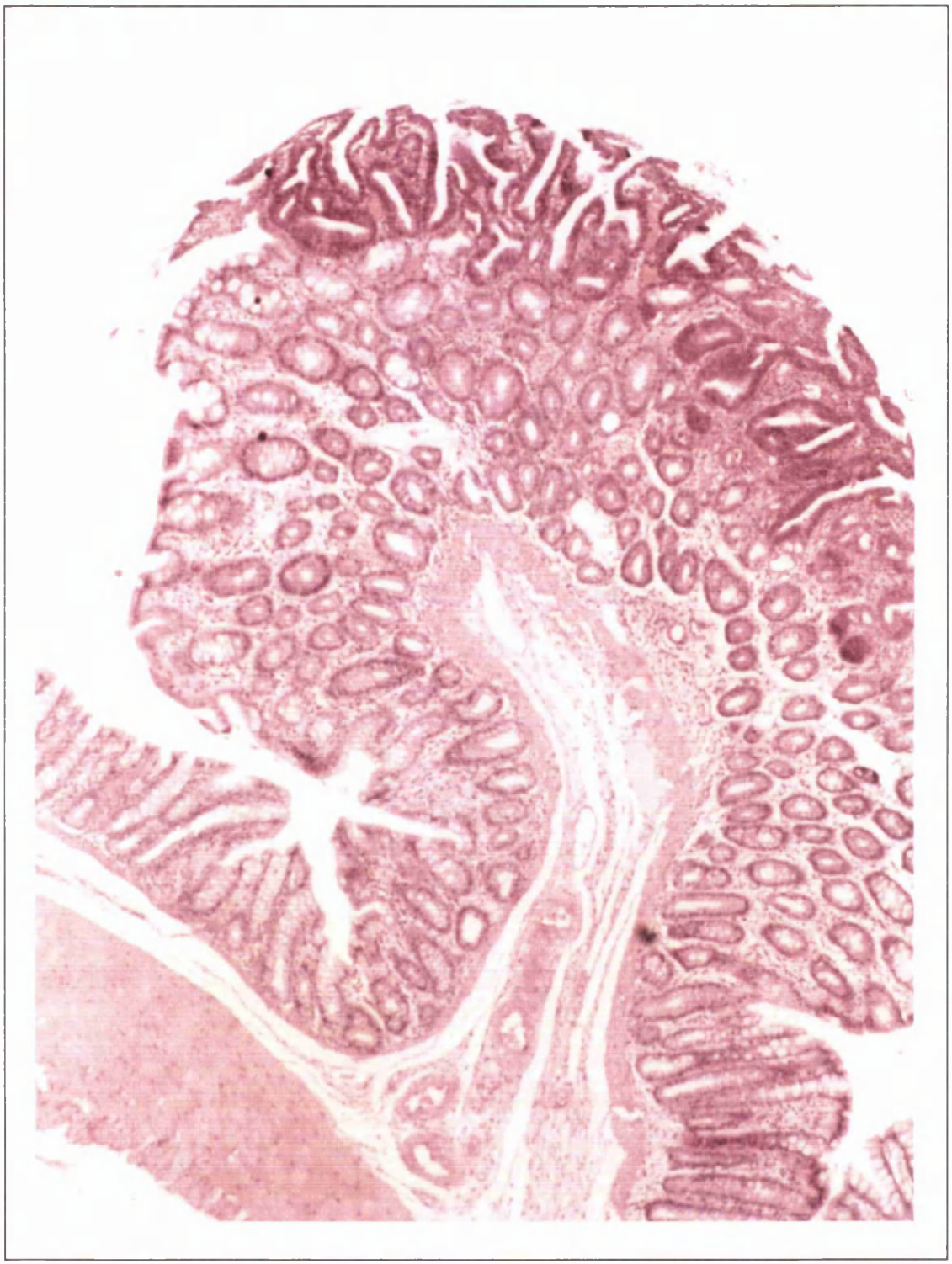


Figure 9.6
Example of an azoxymethane induced benign adenoma (with superficial dysplasia) in a rat treated with omeprazole.

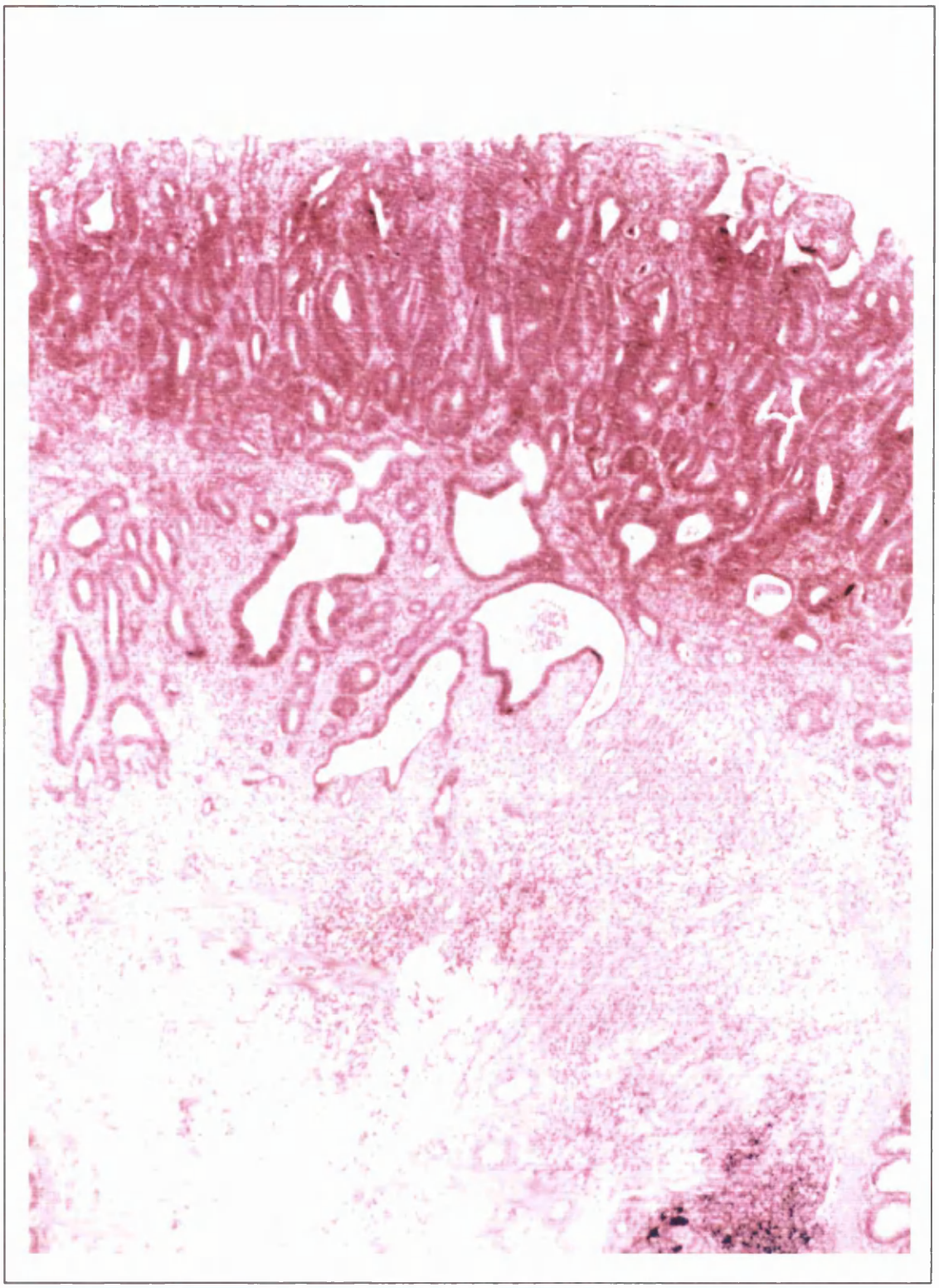


Figure 9.7
Example of an azoxymethane-induced group 2 carcinoma in a rat treated with vehicle.

Group	Mean (± S.D.) colon length (mm)	Median tumour site (% colon length)	Range
Omeprazole	195.94 (±14.74)	32.50	3.00 - 95.00
Vehicle	200.90 (± 16.86)	31.00	3.00 - 70.00

Table 9.11. (2 pages). Tumour site distribution in Omeprazole and Vehicle groups.

		Lesion Number									
Rat No.	**Colon Length (mm)**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1	n/a										
2	210	200 (95)	52 (25)								
3	215	80 (37)									
4	195	20 (10)	31 (16)	45 (23)	120 (61)						
5	195										
6	190	16 (8)	80 (42)	83 (44)							
7	185	37 (20)	70 (38)	75 (41)							
8	*										
9	205	10 (5)									
10	190	10 (5)									
11	185										
12	195										
13	190	40 (20)	81 (44)								
14	n/a										
15	225	7 (3)	12 (5)	43 (19)	90 (40)	104 (46)					
16	n/a										
17	165										
18	180	50 (28)									
19	210	7 (3)	81 (39)	90 (43)	100 (48)						
20	200	90 (45)									

Table 9.12a. Individual tumour site distribution (mm from anorectal margin) in Omeprazole group.
Figures in parentheses represent percentage distance along colon (see text). * Rat excluded. n/a = no result.

		Lesion Number									
Rat No.	**Colon Length (mm)**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1	200	15 (7)	20 (10)	25 (12)	32 (16)	40 (20)	62 (31)	70 (350)	73 (36)	75 (37)	85 (42)
2	200	25 (12)	45 (22)	80 (400)							
3	200	35 (17)	75 (37)	80 (40)							
4	190	30 (16)	44 (23)								
5	195	22 (11)	26 (130)	48 (25)	73 (37)	95 (49)					
6	175	42 (24)	71 (41)	80 (46)							
7	190	11 (6)	22 (12)	51 (27)	80 (42)						
8	180	56 (31)	70 (39)	83 (46)							
9	205	15 (7)	48 (23)	72 (35)							
10	195	20 (10)	30 (15)	73 (37)	82 (42)						
11	230	80 (35)									
12	205										
13	205	7 (3)									
14	190	15 (8)									
15	175	30 (17)	73 (42)								
16	220	58 (26)									
17	190	20 (11)	26 (14)	80 (42)	80 (42)						
18	230	12 (5)	45 (20)	110 (48)	120 (52)	145 (63)					
19	230	85 (37)	160 (70)								
20	210	100 (48)	125 (60)								

Table 9.12b. Individual tumour site distribution (mm from anorectal margin) in Vehicle group.
Figures in parentheses represent percentage distance along colon (see text). * Rat excluded. n/a = no result.

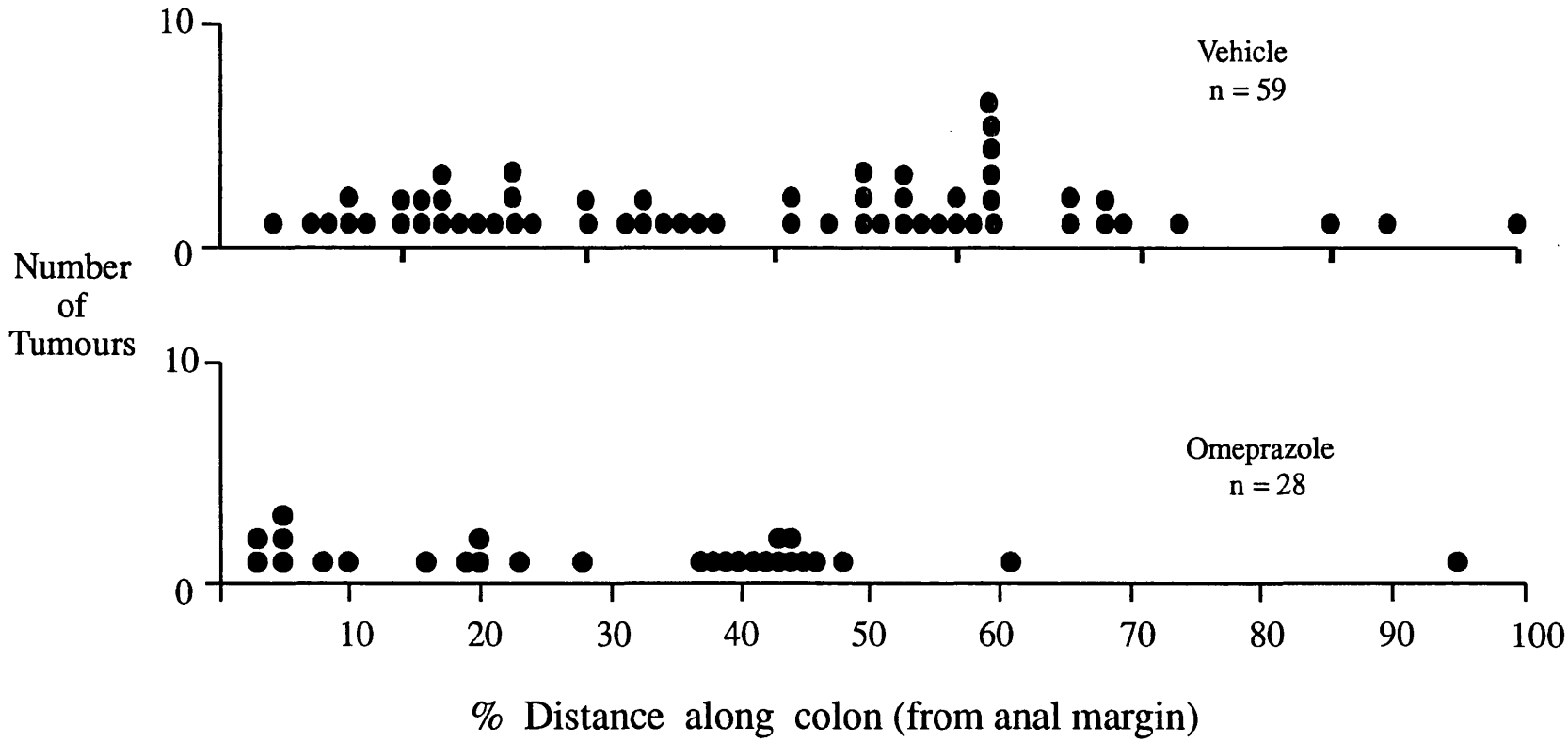


Figure 9.8

Site distribution of azoxymethane-induced tumours, expressed as a percentage distance along colon (0 = anorectal margin, 100 = caecum).

Group	Number of tumours	Median tumour volume (mm^3)	Range
Omeprazole	28	27.00	1.00 - 288.00
Vehicle	59	30.00	1.00 - 800.00

Table 9.13. Colorectal tumour volumes (mm^3).

Lesion Number

Rat No.	1	2	3	4	5	6	7	8	9	10
1										
2	27	27								
3	18									
4	80	60	36	24						
5										
6	12	12								
7	2	8	27	48						
8										
9	192									
10	45									
11										
12										
13	15	1								
14										
15	48	18	36	18	18					
16										
17										
18	225									
19	6	48	48	288						
20	140									

Table 9.14a. Individual tumour volumes (mm^3) in Omeprazole group.

Lesion Number

Rat No.	1	2	3	4	5	6	7	8	9	10
1	30	252	30	36	112	6	4	12	480	30
2	6	30	600							
3	8	24	75							
4	1	1								
5	1	1	8	120	294					
6	48	8	125							
7	8	8	45	60						
8	18	18	8							
9	1	2	4							
10	1	8	300	800						
11	300									
12										
13	36									
14	150									
15	80	60								
16	30									
17	8	8	8	360						
18	8	48	48	120	250					
19	75	96								
20	75	18								

Table 9.14b. Individual tumour volumes (mm^3) in Vehicle group.

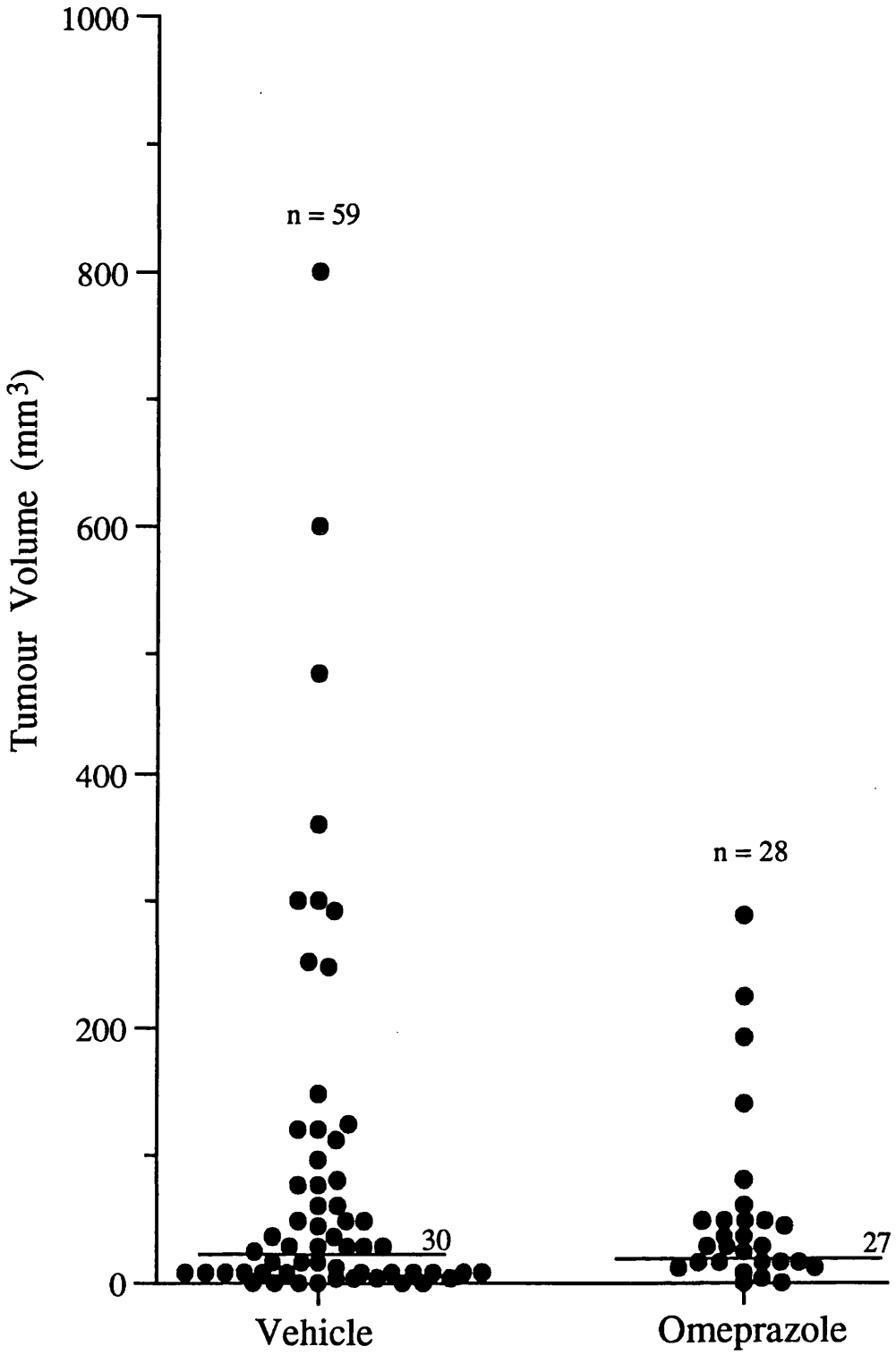


Figure 9.9

Individual tumour volumes (mm^3). Horizontal bars indicate median values.

9.5 DISCUSSION

The aim of this study was to assess whether persistent endogenous hypergastrinaemia secondary to prolonged omeprazole treatment would enhance colorectal carcinogenesis induced in rats by azoxymethane. Despite causing marked hypergastrinaemia (levels raised up to ten-fold) the results show that, contrary to the hypothesis, chronic omeprazole therapy significantly inhibited tumour formation. There are several possible explanations for this observation.

Firstly, it is possible that omeprazole-treated rats developed tumours with similar frequency but that the drug inhibited their growth, resulting in many microscopic lesions being missed in the omeprazole group. This is unlikely because the samples of normal flat colon taken from all rats failed to reveal any early microscopic lesions. Furthermore, if omeprazole inhibited the growth of tumours once they developed, then one would have expected this group to have on average smaller tumours but this was not the case. It is possible to speculate, however, that omeprazole may have exerted its effects on tumour initiation by the mechanisms discussed below but that once tumours developed the trophic effects of the elevated gastrin levels in this group resulted in more rapid tumour growth with the final result being an overall similarity in the measured tumour volumes in the two groups. Further experiments using antrectomised rats or doses of omeprazole which do not significantly raise gastrin levels would be needed to dissociate the effects of the drug *per se* from those due to gastrin. Finally, Graffner *et al* (1992) did not find any effect of omeprazole on the growth of colonic tumour xenografts in mice and so inhibitory effects of omeprazole on tumour growth seem unlikely.

Secondly, underfeeding and/or calorie restriction with reduced weight gain and growth have been shown to inhibit the development of tumours at various sites, including the colon, in animals (Albanes 1987; Kritchevsky *et al* 1990). In the present study, the omeprazole-treated animals did grow more slowly than the vehicle-treated rats and were on average 12% lighter at the end of the study. Calculated food intake measured frequently throughout the experiment (Table 9.2) did not show that rats in the

omeprazole group consumed less - in fact food intake was either similar or *greater* in the omeprazole group at four of the five time points measured. These findings of increased food intake but slightly reduced weight gain are entirely consistent with what was noted during the development and testing of omeprazole (Ekman *et al* 1985) although it is not known why it occurs. When the nineteen omeprazole rats included in effective numbers for analysis were subdivided into those with tumours (n = 12) and those without tumours (n = 7) there was no significant difference between their mean weights (Mann Whitney U test). Using Spearman's rank correlation coefficient there was no correlation between final weight and the number of tumours per rat ($\rho_S = 0.195$; $P > 0.2$) or between animal weight and the total tumour "burden" per rat, "burden" being the total volume of all lesions in that rat ($\rho_S = -0.149$; $P > 0.2$). Furthermore, the study of Kumar *et al* found significant inhibition of azoxymethane-induced tumours only at levels of calorie restriction of 20% or more (Kumar *et al* 1990) and from other published data on calorie restriction and experimental carcinogenesis (Kritchevsky *et al* 1990; Albanes 1987), the minor weight difference in this study is unlikely to have made more than a minor contribution to the results.

An alternative possibility for the reduced tumour incidence in the omeprazole group is that the gastrin levels were excessively high and may even have inhibited cell growth. The levels of gastrin reported to stimulate colonic epithelial proliferation vary greatly. While modest two to three-fold rises in gastrin have been found to be trophic, even G-17 concentrations as high as 1600pmol.L^{-1} (equivalent to 3200ng.L^{-1}) stimulated a colorectal cancer cell line *in vitro* (Yactayo *et al* 1991) although maximal stimulation occurred at lower doses (400pmol.L^{-1} i.e. 800ng.L^{-1}). Durrant *et al* also found that the optimum concentration of gastrin for several cell lines was 3ng/ml (i.e. 3000ng.L^{-1}) when testing gastric and colorectal cancer cell lines for their trophic responses to added gastrin (Durrant *et al* 1991). The maximum stimulation of HT29 colon cancer cells in one study occurred in response to 400pmol.L^{-1} of G-17 (equivalent to 800ng.L^{-1}) but even a dose of 4000pmol.L^{-1} (8000ng.L^{-1}) stimulated growth, albeit to a lesser degree (Smith *et al* 1988). In addition the same authors found

that very high doses of pentagastrin (500 and 1000 $\mu g.kg^{-1}$) stimulated the growth of colonic cancer xenografts in mice.

In a study of thirty-one fresh human colorectal tumours, screened *in vitro* for their trophic responsiveness to gastrin (Watson *et al* 1989b), seventeen of the thirty-one tumours were found to be gastrin responsive, the majority responding maximally to high concentrations of gastrin (2-50$\mu g.L^{-1}$ i.e. 2000-50000$ng.L^{-1}$). Normal rat colonic epithelium *in vivo* has been found to respond trophically to doses of pentagastrin ranging up to 1000$\mu g.kg^{-1}$ with a significant quadratic dose response relationship (Fatemi *et al* 1984) and a similar dose response relationship for gastrin on gastric ECL cells has also been found (Brenna *et al* 1993). In this study constant infusions of gastrin were trophic to the ECL cells with the maximal effect occurring at circulating gastrin levels of 250-400$pmol.L^{-1}$ (equivalent to 500-800$ng.L^{-1}$, a seven to eleven-fold elevation of gastrin levels compared to control animals). Even the highest infusion dose of gastrin, which resulted in a fourteen-fold elevation in serum gastrin levels, was trophic to the cells. Thus, the levels of gastrin obtained in this thesis are similar to those which have previously been demonstrated to be trophic for rat gastrointestinal epithelium.

The gastrin levels in the rats were comparable to those which may be seen during chronic omeprazole therapy in man. While the majority of patients have three or four-fold rises in gastrin, in the study by Jansen *et al* eight out of thirty-two patients during longterm omeprazole treatment had *fasting* gastrin levels of over 500 $ng.L^{-1}$ which was more than ten times the median level (Jansen *et al* 1990). Bearing in mind that the postprandial rise in gastrin is maintained during omeprazole treatment, their postprandial levels would be more than 1000$ng.L^{-1}$. Also, pernicious anaemia patients may have gastrin levels of around 2000-4000$ng.L^{-1}$ (Lamers 1980). Thus, the hypergastrinaemia produced in the current study is relevant to the levels seen clinically in patients with pernicious anaemia and also during omeprazole therapy.

It therefore seems unlikely that the above reasons are unlikely to explain the reduced prevalence of tumours in the omeprazole group and an effect of omeprazole itself on the carcinogenic process must be considered. Longterm therapy with acid

inhibitory drugs has been shown to result in bacterial overgrowth in the upper gastrointestinal tract (Sharma *et al* 1984), altering the composition of the intestinal microflora. Such bacteria may be important in colonic carcinogenesis and contribute to the tumourigenicity of azoxymethane (Reddy *et al* 1974), possibly by participating in metabolism of the carcinogen in the colon and it is conceivable that the omeprazole effect on colonic carcinogenesis was secondary to the drug affecting the composition and enzymatic activity of the intestinal microflora.

Alternatively, omeprazole may have exerted its effects by modifying the metabolism of azoxymethane, thereby reducing its effectiveness. Most known carcinogens exist as procarcinogens and require metabolic activation, usually by hydroxylation in the liver, in order to exert their genotoxic effects (Guengerich 1988; Snyderwine *et al* 1992) and these reactions are carried out by members of the cytochrome P450 supergene family of enzymes (Gonzalez and Gelboin 1991). Cytochromes P450 IA1 and IA2 (CYPIA1 and CYPIA2) have been found to be the main ones involved in the metabolic activation of a variety of carcinogens including numerous heterocyclic amines commonly present in cooked food (Turesky *et al* 1991; Wakabayashi *et al* 1992) and polycyclic aromatic hydrocarbons. The levels of expression of these enzymes, and their activities, both in the liver and also in extrahepatic tissues such as small intestine and colon can be modified by various factors. These include dietary lipid (Yoo *et al* 1992), extracts of cruciferous vegetables (Vang *et al* 1991) and xenobiotics including other carcinogens (Harper *et al* 1990; Kleman *et al* 1990). It is possible that the relative expressions of these and other P450 enzymes in different tissues may be important in determining the overall balance between activation and detoxification of a given xenobiotic and its carcinogenic effectiveness. Cytochrome P450 enzymes capable of metabolising azoxymethane have also been found in the colon of humans and animals (Stralka and Strobel 1991; Rosenberg 1991; White *et al* 1991). While CYP IIE1 appears to be important in the activation of azoxymethane (Sohn *et al* 1991), the full pathway of azoxymethane metabolism has not been elucidated and the role of other cytochrome P450 enzymes, notably CYP IA1 and IA2 remains to be determined.

Omeprazole has been shown to interact with the cytochrome P450 system. In 1990, Diaz *et al* (1990) showed that omeprazole was a potent inducer of both CYPIA1 and IA2 in human hepatocytes *in vitro* (although no effect CYPIIE1 was noted) and raised fears that this may result in *increased* activation of carcinogens metabolised by these enzymes. Subsequently the newer H^+/K^+ ATPase inhibitors, pantoprazole and lansoprazole, have also been found to modulate cytochrome P450 enzyme activity in animals (Simon *et al* 1991). Also, McDonnell *et al* (1992) have recently demonstrated that omeprazole treatment for seven days induced CYP IA1 gene expression and enzyme activity *in vivo* in endoscopic biopsies from human volunteers with the most marked effects seen in the duodenum. These are probably direct effects of the drug or a metabolite rather than the resulting hypergastrinaemia as continuous infusions of pentagastrin do not seem to affect cytochrome P450 enzyme activity, at least in the small intestine (Pascoe and Correia 1988).

These findings and the concerns expressed about omeprazole have proved controversial and stimulated much debate (Farrell and Murray 1990; Lucier *et al* 1992; Parkinson and Hurwitz 1991; Moldeus *et al* 1991). While it appears logical to assume that induction of CYP IA proteins, with increased activation of heterocyclic amines and increased mutagenicity, would result in enhanced carcinogenicity, this has not been found to be the case when studied in animal models of cancer development. Indeed the contrary has been found to be the case, with induction of IA proteins being repeatedly shown to be associated with *protection* against tumour formation (Anderson and Seetharam 1985; Miller *et al* 1958; Wattenberg and Leong 1970). Similarly, inhibition of these enzymes is associated with enhanced carcinogenesis in animals (Wheatley 1968), despite the reduced activation of the carcinogens under study. Thus, demonstrating increased carcinogen activation and mutagenicity *in vitro* on the one hand does not necessarily correlate with increased tumour formation. In fact the opposite seems to apply, highlighting our relative lack of understanding of this extremely complex subject.

The reduced carcinogenic activity of azoxymethane during omeprazole therapy may be related to the latter modifying the metabolism of the carcinogen in the colon or

liver or affecting P450 isoenzymes differentially at these two sites. Further studies on both the pathway of azoxymethane metabolism and the effects of omeprazole on this pathway are obviously needed before conclusions can be made regarding the mechanism(s) by which omeprazole exerts its protective effects. For instance, it is not known whether the omeprazole-inducible enzymes IA1 and IA2 are important in azoxymethane metabolism.

Due to the unexpected inhibitory effects of omeprazole on colorectal carcinogenesis, the present model is unsuitable for assessing the effect of chronic hypergastrinaemia on colonic carcinogenesis. Chronic hypergastrinaemia due to other causes, however, cannot be excluded as important in colorectal cancer development. The present studies do suggest that fears regarding the potential deleterious effects of omeprazole on carcinogenesis (Diaz *et al* 1990; Simon *et al* 1991; McDonnell *et al* 1992) may be unfounded. Further studies on the mechanism(s) by which omeprazole protects against chemical colorectal tumourigenesis and the doses at which this occurs are merited and may shed light on the pathogenesis of this common disease.

CHAPTER 10

PLASMA GASTRIN CONCENTRATIONS IN HUMAN COLORECTAL NEOPLASIA

10.1 INTRODUCTION

Some authors have reported that patients with conditions which result in hypergastrinaemia (such as pernicious anaemia and following truncal vagotomy or Billroth I gastrectomy) are at increased risk of subsequent development of colorectal cancer (Bundred *et al* 1985; Talley *et al* 1989; Stemmermann *et al* 1991) although others have not confirmed these findings (Kune *et al* 1988; Brinton *et al* 1989). In view of this, and the results of experimental work supporting a possible role for gastrin in colorectal cancer, several studies have examined circulating gastrin concentrations in patients with such tumours. In 1989 Smith *et al* (1989) reported that a proportion of patients with colonic polyps and carcinomas have elevated circulating gastrin levels. Since then at least four studies have confirmed this finding (Seitz *et al* 1989; Wong *et al* 1991; Seitz *et al* 1992; Charnley *et al* 1992) but a similar number have found no evidence of hypergastrinaemia in tumour patients compared to controls (Suzuki *et al* 1988; Kaufmann and Ottenjann 1991; Yapp *et al* 1992; Kikendall *et al* 1992; Scotté *et al* 1992). Differences in study design and selection of patients and controls, along with differing methods of statistical analysis of the results may account for these conflicting results. In particular, none of the above studies has controlled for the presence of gastric colonisation with *Helicobacter pylori* (HP), a common infection resulting in chronic gastritis and significantly raised fasting and meal-stimulated gastrin levels which fall after eradication of the organism (McColl *et al* 1989). The need for further carefully controlled studies of gastrin in patients with colorectal neoplasia has recently been stressed (Boland 1991; Wolfe 1992).

The aim of the present study was to compare fasting and meal-stimulated plasma gastrin levels in colorectal tumour patients and in closely matched control patients. In addition we proposed to assess whether resection of the tumour altered the gastrin concentration.

10.2 MATERIALS AND METHODS

10.2.1 Patients

Forty-two newly diagnosed patients with histologically confirmed sporadic colorectal neoplasia and who were in hospital awaiting elective surgery were studied. Forty patients had carcinomas and two had large (≥ 5cm), severely dysplastic adenomas. These forty-two patients were selected from a total of one hundred and twenty who were initially considered for the study but excluded because they were too unwell or because of one or more of the factors outlined below. Patients with familial adenomatous polyposis, intestinal obstruction or requiring emergency surgery were excluded. Thirty-four age- and sex- matched inpatients, awaiting elective surgery under general anaesthesia for relatively minor conditions (for example inguinal hernia, varicose veins, haemorrhoids) and with no history of intestinal disease served as controls. These patients were chosen as controls in preference to healthy volunteers in order to control for any possible effects that the stress of hospitalisation and waiting for surgery may have on gastrin levels. Those with a history of malignancy at any other site, treatment with acid suppressing drugs (H_2 antagonists or proton pump inhibitors), previous peptic ulcer surgery, documented pernicious anaemia, renal failure, hypercalcaemia, recent antibiotic therapy (within one week) or any form of bowel preparation in the two days prior to the tests were excluded from study. Informed written consent was obtained from all patients and the studies were approved by the Western Infirmary Ethical Committee. Patient details are given in Tables 10.1 - 10.3.

10.2.2 Measurement of Basal and Meal-Stimulated Gastrin Levels

All studies were performed between 0900 and 1000 after subjects had fasted overnight. A 16F gauge cannula attached to a three-way tap was inserted into an antecubetal vein and kept patent with heparinised saline. Measurement of meal-stimulated gastrin concentrations and the [^{14}C]urea breath test for *H. pylori* status were performed simultaneously and the schedule for both tests is schematically shown in Figure 10.1.

	Tumours	Controls
NUMBER	42	34
AGE		
mean	68.3	65.7
S.D.	11.25	13.5
median	69.5	67.5
range	44-93	36-88
SEX		
male	25 (60%)	20 (59%)
female	17 (40%)	14 (41%)

Table 10.1. Patient characteristics.

Case No.	Initials	Age	Sex	Tumour site	Operation	Curative resection (Y/N)	Histology	Different-iation	Dukes stage	Site of metastases
502088	G.A.	67	F	rectum	anterior resection	Y	adenocarcinoma	moderate	B	
322234	J.A.	70	M	rectum	anterior resection	Y	adenocarcinoma	moderate	B	
835887	H.B.	66	M	sigmoid	sigmoid colectomy	N	adenocarcinoma	poor	D	liver
242117	J.B.	59	M	rectosigmoid	anterior resection	N	adenocarcinoma	moderate	C	
936325	D.BU.	61	F	rectum	anterior resection	Y	adenocarcinoma	moderate	C	
251770	I.C.	78	M	caecum	right hemicolectomy	N	adenocarcinoma	moderate	C	
649010	J.CO.	70	M	sigmoid	sigmoid colectomy	Y	adenocarcinoma	moderate	B	
847494	A.C.	53	F	rectum	AP resection	N	adenocarcinoma	moderate	D	liver
936921	J.C3.	46	M	rectum	anterior resection	Y	adenocarcinoma	moderate	C	
855961	I.D.	67	F	sigmoid	pelvic exenteration	N	adenocarcinoma	poor	C	
473785	A.F.	79	F	rectum	colostomy (defunction)	N	adenocarcinoma	moderate	D	liver

Table 10.2. (4 pages). Clinical and pathological details of colorectal tumour pateints. Y = yes, N = no. AP = abdominoperineal.

Case No.	Initials	Age	Sex	Tumour site	Operation	Curative resection (Y/N)	Histology	Different-iation	Dukes stage	Site of metastases
454435	J.G.	78	M	rectum	local excision	Y	villous adenoma	---	---	
480507	E.G.	52	F	caecum	right hemicolectomy	Y	adenocarcinoma	moderate	C	
497088	J.H.	47	M	rectum	anterior resection	Y	adenocarcinoma	moderate	A	
121591	M.H.	66	F	descending colon	left hemicolectomy	Y	adenocarcinoma	moderate	B	
516043	A.J.	79	M	caecum	right hemicolectomy	Y	adenocarcinoma	well	B	
738266	J.K.	67	M	rectum	AP resection	N	adenocarcinoma	moderate	C	
62502	A.L.	93	F	rectum	anterior resection	Y	adenocarcinoma	moderate	C	
222730	P.L.	75	M	rectum	anterior resection	Y	adenocarcinoma	moderate	B	
850663	I.M.	64	F	rectum	anterior resection	Y	adenocarcinoma	moderate	C	
542878	I.McC.	79	M	rectum	anterior resection	N	adenocarcinoma	poor	D	liver
657891	J.McD.	76	F	sigmoid	sigmoid colectomy	N	adenocarcinoma	moderate	C	

Table 10.2. contd. (page 2 of 4). Clinical and pathological details of colorectal tumour pateints. Y = yes, N = no. AP = abdominoperineal.

Case No.	Initials	Age	Sex	Tumour site	Operation	Curative resection (Y/N)	Histology	Different-iation	Dukes stage	Site of metastases
402383	M.McD.	66	F	rectum	anterior resection	N	adenocarcinoma	moderate	D	liver
165739	W.McI.	79	M	rectum	AP resection	Y	adenocarcinoma	moderate	C	
394247	G.McK.	71	M	caecum	right hemicolectomy	N	adenocarcinoma	poor	D	liver
443246	T.McL.	58	M	rectum	anterior resection	Y	adenocarcinoma	moderate	C	
474018	R.McN.	75	M	rectosigmoid	colostomy (defunction)	N	adenocarcinoma	poor	C	
928284	E.McN.	54	F	rectum	anterior resection	N	adenocarcinoma	moderate	D	liver
564327	N.McR.	54	M	rectosigmoid	anterior resection	N	adenocarcinoma	moderate	D	liver
709277	M.McR.	80	F	rectum	colostomy (defunction)	N	adenocarcinoma	moderate	D	liver
810870	M.M.	56	M	rectum	anterior resection	N	adenocarcinoma	moderate	D	liver
410814	G.N.	68	M	sigmoid	sigmoid colectomy	Y	adenocarcinoma	moderate	B	
636073	M.P.	65	F	caecum	right hemicolectomy	Y	adenocarcinoma	moderate	B	

Table 10.2. contd. (page 3 of 4). Clinical and pathological details of colorectal tumour pateints. Y = yes, N = no. AP = abdominoperineal.

Case No.	Initials	Age	Sex	Tumour site	Operation	Curative resection (Y/N)	Histology	Different-iation	Dukes stage	Site of metastases
817655	I.R.	72	M	descending colon	Nil	Y	tubular adenoma	---	---	
807750	M.R.	75	F	caecum	right hemicolectomy	Y	adenocarcinoma	well	B	
929989	H.S.	72	F	caecum	right hemicolectomy	N	adenocarcinoma	moderate	D	liver
562802	A.S.	79	F	caecum	right hemicolectomy	Y	adenocarcinoma	moderate	C	
922552	J.S.	80	M	rectum	anterior resection	N	adenocarcinoma	moderate	C	
372926	B.T.	44	M	sigmoid	anterior resection	N	adenocarcinoma	moderate	C	
632272	T.T.	87	M	sigmoid	anterior resection	Y	adenocarcinoma	moderate	B	
850891	C.W	71	M	rectum	anterior resection	Y	adenocarcinoma	moderate	B	
440457	T.W.	63	M	rectum	anterior resection	Y	adenocarcinoma	moderate	B	

Table 10.2. contd. (page 4 of 4). Clinical and pathological details of colorectal tumour pateints. Y = yes, N = no. AP = abdominoperineal.

Case No.	Initials	Age	Sex	Diagnosis
338439	D.A.	66	M	Anal Fissure
328904	W.BR.	75	M	Inguinal hernia
349280	A.C2	65	F	Varicose veins
543298	A.CH	59	M	Pilonidal sinus
857008	H.C.	52	M	Inguinal hernia
502346	M.C2.	79	F	Rectal prolapse
436192	T.C.	71	M	Inguinal hernia
673591	J.C.	42	M	Hydrocoele
99707	J.C2.	75	M	Haemorrhoids
298909	M.C3.	68	F	Lipoma
66109	J.DOC.	7I	M	Inguinal hernia
485107	J.DU.	88	F	Rectal prolapse
570157	E.E.	46	F	Gallstones
922689	J.F.	56	M	Incisional hernia
850006	J.F2.	73	M	Gallstones
532357	C.G.	63	F	Varicose veins
698715	H.H.	72	M	Inguinal hernia

Table 10.3. (2 pages). Clinical and preoperative details of control patient group.

Case No.	Initials	Age	Sex	Diagnosis
806016	O.I.	65	F	Gallstones
n/a	P.K.	60	M	Haemorrhoids
650775	J.M.	36	M	Haemorrhoids
836047	G.M.	74	M	Inguinal hernia
593439	L.M.	85	M	Inguinal hernia
317466	H.McB.	76	F	Varicose veins
426207	J.McG.	46	M	Inguinal hernia
754914	S.McI.	77	F	Skin cyst
933860	W.McL.	72	M	Inguinal hernia
640994	P.McP.	39	M	Inguinal hernia
874293	A.M.	49	M	Inguinal hernia
707804	M.P.	87	F	Ovarian cyst
468860	S.R.	64	F	Ovarian cyst
613277	H.S2.	74	F	Adhesions
741481	M.T.	80	F	Rectal prolapse
504402	B.T2	64	F	Lipoma
643689	W.T.	66	M	Haemorrhoids

Table 10.3 contd. (page 2 of 2). Clinical and preoperative details of control patient group.

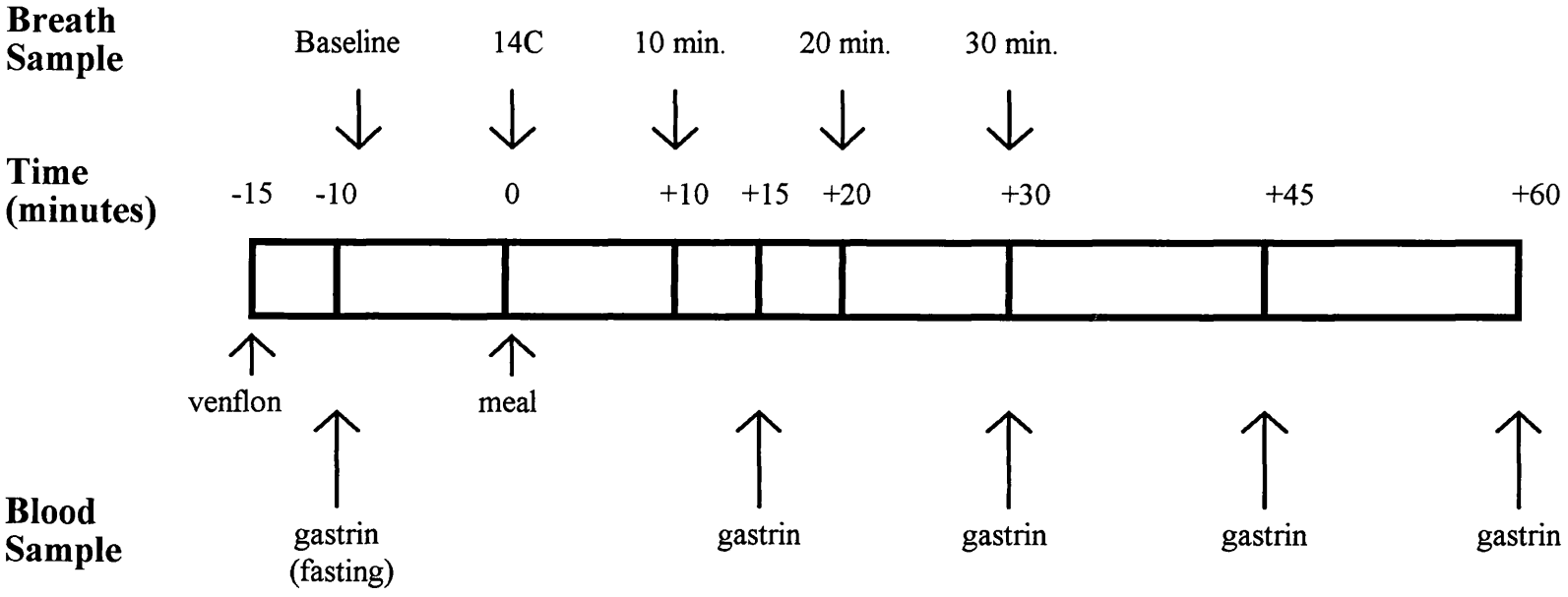


Figure 10.1. Schematic representation of study design.

Blood samples (10ml lithium heparin tube) were taken fasting (00 minutes) and at 15, 30, 45 and 60 minutes following a test meal. The test meal consisted of 150ml of a high lipid drink ("Ensure plus", Abbott Laboratories Ltd., Maidenhead, UK) followed by 200mls of a beef-extract drink consisting of 2 "oxo" cubes (Brooke Bond Foods Ltd., Croydon, UK) dissolved in water at 50°C. Blood samples were centrifuged at 3000 rpm at 4°C for 10 minutes and plasma stored at -70°C until required for analysis. Plasma gastrin was measured in duplicate by an in-house radioimmunoassay using antibody R98 as previously described (Ardill 1973). This antibody recognises both human G-17 and G-34 in equimolar concentrations with a sensitivity of $3ng.L^{-1}$ and does not cross-react with cholecystokinin.

10.2.3 Determination of *Helicobacter pylori* Status

The presence or absence of gastric colonisation with HP was confirmed in all patients using the [^{14}C]urea breath test as previously described (Marshall and Surveyor 1988). Briefly, this test utilises the production of urease enzyme by HP as a means of detecting the organism. Patients swallow 0.4MBq of urea isotopically labelled with ^{14}C and if HP is present the urease hydrolyses the urea to produce $^{14}CO_2$. By collecting breath samples at different times the presence of exhaled $^{14}CO_2$ can be detected using a liquid scintillation counter. This test has been shown to be a sensitive and reliable non-invasive method of diagnosing the presence of HP (Atherton and Spiller 1994). The standard protocol used in the Western Infirmary, Glasgow is given in Appendix 4. In addition serum antibodies (IgG) to the bacterium were measured using a commercial enzyme immunoassay kit (Helico-G™, Porton Cambridge, UK) with a sensitivity of 95% and specificity of 88% (Newell *et al* 1988).

10.2.4 Measurement of Gastric AutoAntibodies

An important cause of hypergastrinaemia is autoimmune-type atrophic gastritis (Lamers 1980). As a screening test for this fasting serum from all patients was analysed for the presence of parietal cell and intrinsic factor (type I) antibodies. The former was assayed

using a commercially available immunofluorescent kit (Zeus Autoantibody Screen Kit, Bayer Diagnostics, Basingstoke, UK) and the latter by a commercial radioassay kit (Immophase™, Blocking Antibody Radioassay, Ciba Corning Diagnostic Corporation, Medfield, MD, USA).

10.2.5 Post-Operative Reassessment

Twenty-five colorectal tumour patients were reassessed at a median of 79 days post-operatively (range 11-213 days). Of the seventeen patients not seen post-operatively four had died, four had inoperable disease, three were too ill with ongoing medical problems and only six were lost to follow-up or refused to return. The tests were carried out at or after hospital discharge once patients had resumed a normal or near-normal lifestyle. They were examined clinically for evidence of tumour recurrence and a careful drug history was taken, including note of any antibiotic therapy prescribed since the time of operation. Patients had repeat measurement of basal and meal-stimulated gastrin levels and reassessment of their HP status by means of the [^{14}C]urea breath test and serology. In all twenty-five of these patients, pre- and post-operative plasma gastrin samples were analysed together in the same assay to exclude interassay variation as a possible source of differences in gastrin levels between the two study days.

10.2.6 Statistics

Differences in HP status and in pernicious anaemia autoantibody prevalence between the groups were assessed using the χ^2-test. Gastrin levels in all groups were not normally distributed (see Results and Discussion) and so non-parametric tests were used throughout: Mann-Whitney U Test for differences between tumours and controls; Wilcoxon signed ranks test (matched pairs) for comparisons of tumour patients pre- and post-operatively; and Kruskal-Wallis for analysis of gastrin levels by tumour site and stage. Analysis of a possible correlation between timing of post-operative reassessment and gastrin levels was performed using Spearman's rank correlation coefficient. A *P*

value of < 0.05 was considered significant. Results are presented as medians and inter-quartile ranges unless otherwise stated.

10.3 RESULTS

10.3.1 Gastrin Levels: Patients versus Controls

Figures 10.2 and 10.3 show the preoperative pattern of meal-stimulated gastrin responses in tumour patients and controls, as well as fasting and peak plasma gastrins in both groups. Median fasting gastrin in preoperative tumour patients was 55ng.L^{-1} (45-82.5) and this was not significantly different from that of 77.5ng.L^{-1} (53.7-137.5) in control patients ($P = 0.10$, Mann Whitney). Similarly, median peak gastrins in tumour patients (200 ng.L^{-1}, 137.5-312.5) and control patients (247.5ng.L^{-1}, 147.5-375) were similar ($P = 0.21$, Mann Whitney). Table 10.4 shows individual meal-stimulated gastrin concentrations in the two groups.

10.3.2 Gastrin Levels: Pre- and Post- Operative Levels in Tumour Patients

In twenty-five of the forty-two colorectal tumour patients the studies were repeated postoperatively at a median follow up time of 79 days (range 11-213). Of these patients eighteen were considered (on the basis of clinical details, operative findings and histology) to have had a "curative" resection. In addition, of the original twenty-five patients, HP status had changed from positive preoperatively to negative postoperatively in five, including three of the eighteen with a "curative" resection. Thus, data were available on fifteen patients with a presumed curative resection and no change in HP status (Figure 10.4). Their median fasting gastrin was similar before (52.5ng.L^{-1}, 43.8-76.2) and after resection (52.5ng.L^{-1}, 43.8-71.3; $P = 0.27$, Wilcoxon) and, likewise, their median peak levels were similar preoperatively (150ng.L^{-1}, 117.5-240) and postoperatively (162.5ng.L^{-1}, 120-270; $P = 0.30$, Wilcoxon).

The seventeen patients not reassessed post-operatively were similar to the twenty-five who were seen again, with respect to age and sex as well as tumour site,

					preoperative						postoperative				
Case No.	**Initials**	**Age**	**Sex**		**00 mins**	**15 mins**	**30 mins**	**45 mins**	**60 mins**		**00 mins**	**15 mins**	**30 mins**	**45 mins**	**60 mins**
502088	G.A.	67	F		55	255	180	150	n/a		75	330	300	210	90
322234	J.A.	70	M		120	625	500	375	225		50	375	350	300	300
835887	H.B.	66	M		70	170	140	170	200		60	100	125	140	105
242117	J.B.	59	M		55	55	50	40	35						
936325	D.BU.	61	F		45	120	150	105	85		35	180	180	135	135
251770	I.C.	78	M		50	150	85	65	n/a						
649010	J.CO.	70	M		50	110	90	90	65		55	135	210	135	135
847494	A.C.	53	F		120	n/a	n/a	n/a	n/a		110	180	240	255	225
936921	J.C3.	46	M		45	150	210	195	165		60	105	150	105	105
855961	I.D.	67	F		50	95	135	135	135						
473785	A.F.	79	F		120	350	275	300	525						

Table10.4a. (4 pages). Meal-stimulated plasma gastrin concentrations ($ng.L^{-1}$) in tumour patients.

					preoperative						postoperative				
Case No.	**Initials**	**Age**	**Sex**		**00 mins**	**15 mins**	**30 mins**	**45 mins**	**60 mins**		**00 mins**	**15 mins**	**30 mins**	**45 mins**	**60 mins**
454435	J.G.	78	M		45	120	140	105	100						
480507	E.G.	52	F		30	45	60	55	90		45	75	70	85	150
497088	J.H.	47	M		55	150	140	120	110		30	125	140	130	100
121591	M.H.	66	F		30	65	60	105	120		40	75	85	105	120
516043	A.J.	79	M		75	190	200	120	120		80	160	140	120	110
738266	J.K.	67	M		135	300	390	270	330						
62502	A.L.	93	F		45	180	240	180	180		45	180	155	135	180
222730	P.L.	75	M		600	600	500	450	450		750	950	700	700	600
850663	I.M.	64	F		90	285	120	90	n/a						
542878	I.McC.	79	M		120	240	225	240	260						
657891	J.McD.	76	F		80	400	300	325	n/a						

Table10.4a contd. (page 2 of 4). Meal-stimulated plasma gastrin concentrations ($ng.L^{-1}$) in tumour patients.

					preoperative						postoperative				
Case No.	**Initials**	**Age**	**Sex**		**00 mins**	**15 mins**	**30 mins**	**45 mins**	**60 mins**		**00 mins**	**15 mins**	**30 mins**	**45 mins**	**60 mins**
402383	M.McD.	66	F		60	255	345	345	405						
165739	W.McI.	79	M		55	150	110	135	120		40	65	100	65	55
394247	G.McK.	71	M		180	340	400	380	500		180	340	320	340	360
443246	T.McL.	58	M		50	150	120	60	70		55	120	135	165	150
474018	R.McN.	75	M		80	150	150	165	210						
928284	E.McN.	54	F		65	340	260	200	220		80	330	270	240	165
564327	N.McR.	54	M		50	90	85	85	70						
709277	M.McR.	80	F		75	180	240	180	195						
810870	M.M.	56	M		40	95	150	165							
410814	G.N.	68	M		75	180	220	240	240		80	180	260	240	240
636073	M.P.	65	F		140	480	440	440	340		120	240	260	260	220

Table10.4a contd. (page 3 of 4). Meal-stimulated plasma gastrin concentrations ($ng.L^{-1}$) in tumour patients.

					preoperative						postoperative				
Case No.	**Initials**	**Age**	**Sex**		**00 mins**	**15 mins**	**30 mins**	**45 mins**	**60 mins**		**00 mins**	**15 mins**	**30 mins**	**45 mins**	**60 mins**
817655	I.R.	72	M		50	195	255	210	210						
807750	M.R.	75	F		105	315		270	435						
929989	H.S.	72	F		50	55	60	60	55						
562802	A.S.	79	F		30	55	55	60	60		50	85	90	95	120
922552	J.S.	80	M		45	160	90	80	55		55	80	140	100	90
372926	B.T.	44	M		20	35	35	35	35		40	40	40	50	50
632272	T.T.	87	M		80	135	150	135	135		45	70	70	70	55
850891	C.W	71	M		80	130	180	190	n/a		70	210	230	300	250
440457	T.W.	63	M		40	75	100	65	45		65	80	55	85	60

Table10.4a contd. (page 4 of 4). Meal-stimulated plasma gastrin concentrations ($ng.L^{-1}$) in tumour patients.

Case No.	Initials	Age	Sex		00 mins	15 mins	30 mins	45 mins	60 mins
338439	D.A.	66	M		80	195	120	100	55
328904	W.BR.	75	M		75	345	270	225	225
349280	A.C2	64	F		200	625	500	450	450
543298	A.CH	59	M		50	70	70	60	70
857008	H.C.	52	M		25	55	55	50	55
502346	M.C2.	79	F		900	2250	1950	2100	1200
436192	T.C.	71	M		110	240	300	270	240
673591	J.C.	42	M		35	55	65	90	240
99707	J.C2.	75	M		55	340	320	260	240
298909	M.C3.	68	F		145	165	195	210	210
66109	J.DOC.	7I	M		120	300	300	270	240
485107	J.DU.	88	F		150	345	345	270	210
570157	E.E.	46	F		60	255	165	150	105
922689	J.F.	56	M		85	160	380	460	340
850006	J.F2.	74	M		750	1050	750	750	975
532357	C.G.	63	F		325	550	650	900	n/a
698715	H.H.	72	M		120	345	345	285	165

Table10.4b. (2 pages). Meal-stimulated plasma gastrin concentrations ($ng.L^{-1}$) in control patients.

Case No.	Initials	Age	Sex		00 mins	15 mins	30 mins	45 mins	60 mins
806016	O.I.	65	F		85	255	270	210	285
n/a	P.K.	60	M		135	135	120	150	150
650775	J.M.	36	M		180	150	150	210	195
836047	G.M.	74	M		10	50	45	45	45
593439	L.M.	85	M		65	95	150	150	135
317466	H.McB.	76	F		35	80	180	80	80
426207	J.McG.	46	M		105	375	375	300	225
754914	S.McI.	77	F		230	375	375	325	250
933860	W.McL.	72	M		55	95	95	85	70
640994	P.McP.	39	M		25	105	90	90	75
874293	A.M.	49	M		45	195	150	120	105
707804	M.P.	87	F		110	375	250	100	100
468860	S.R.	64	F		70	85	130	140	95
613277	H.S2.	74	F		60	180	240	135	90
741481	M.T.	80	F		225	575	350	275	200
504402	B.T2	64	F		25	95	95	85	70
643689	W.T.	66	M		55	70	90	75	130

Table10.4b contd. (page 2 of 2). Meal-stimulated plasma gastrin concentrations ($ng.L^{-1}$) in control patients.

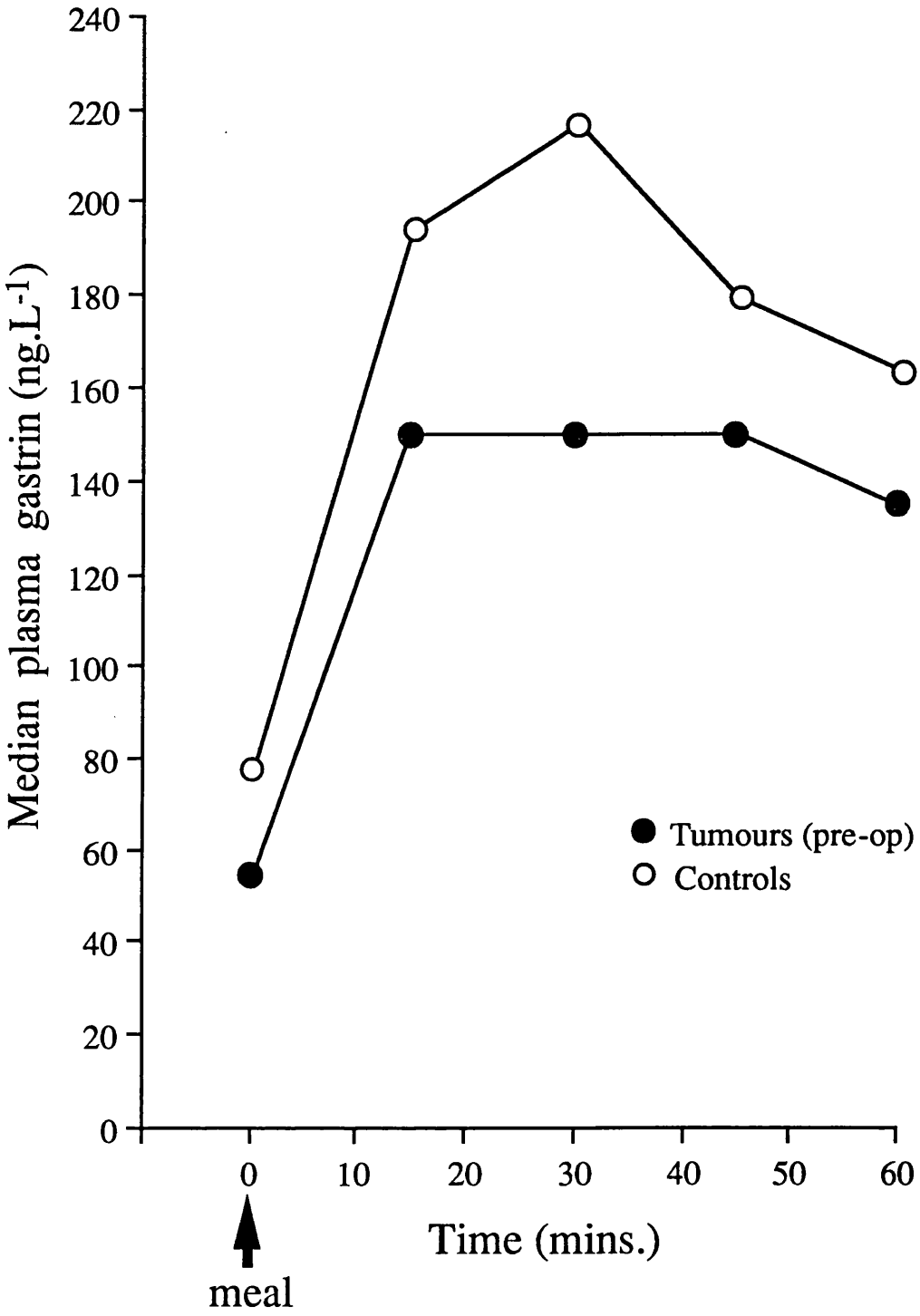


Figure 10.2

Meal-stimulated plasma gastrin concentrations preoperatively in tumour and control patients.

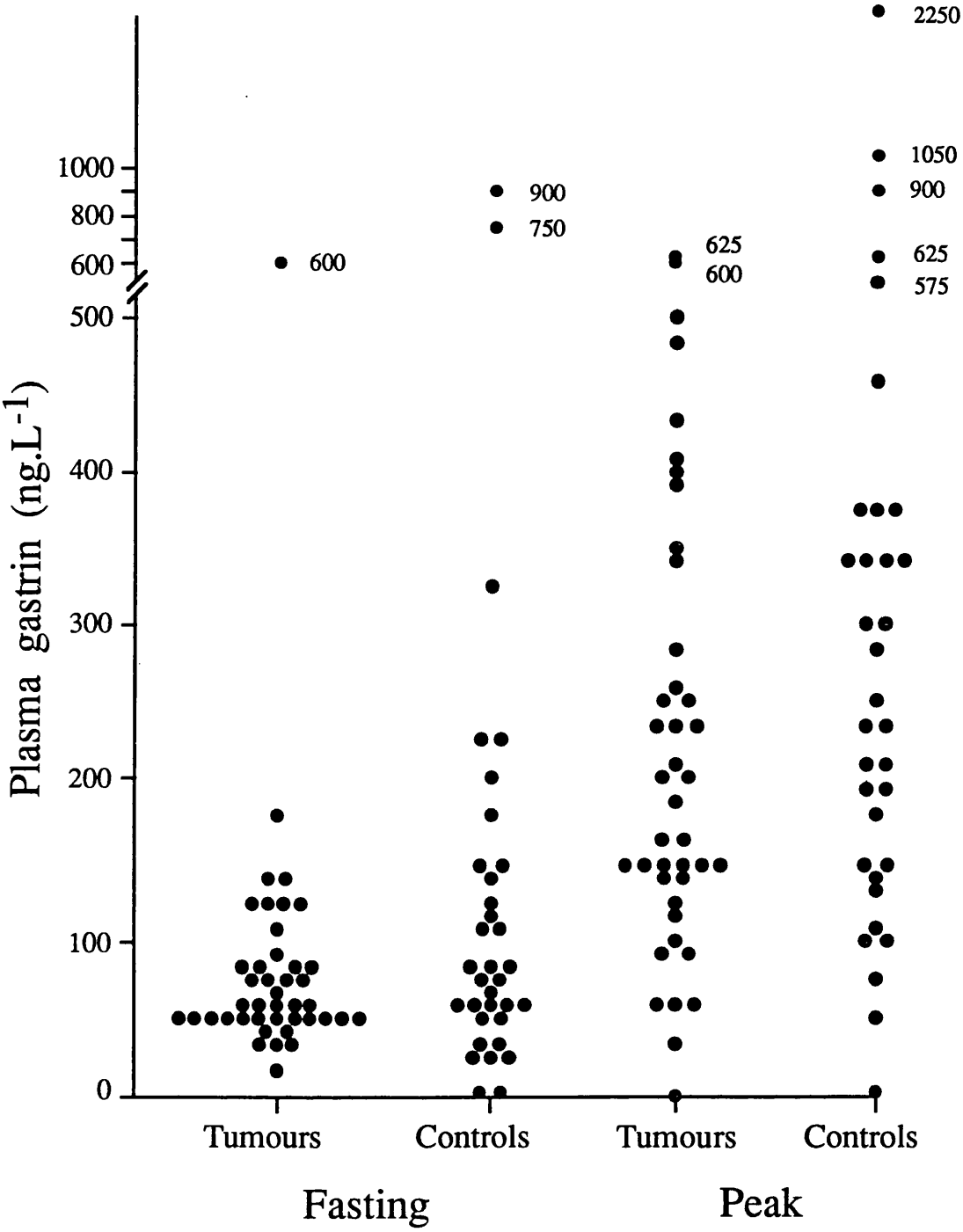


Figure 10.3

Individual fasting and peak plasma gastrin concentrations in tumour and control patients. Note break in Y-axis.

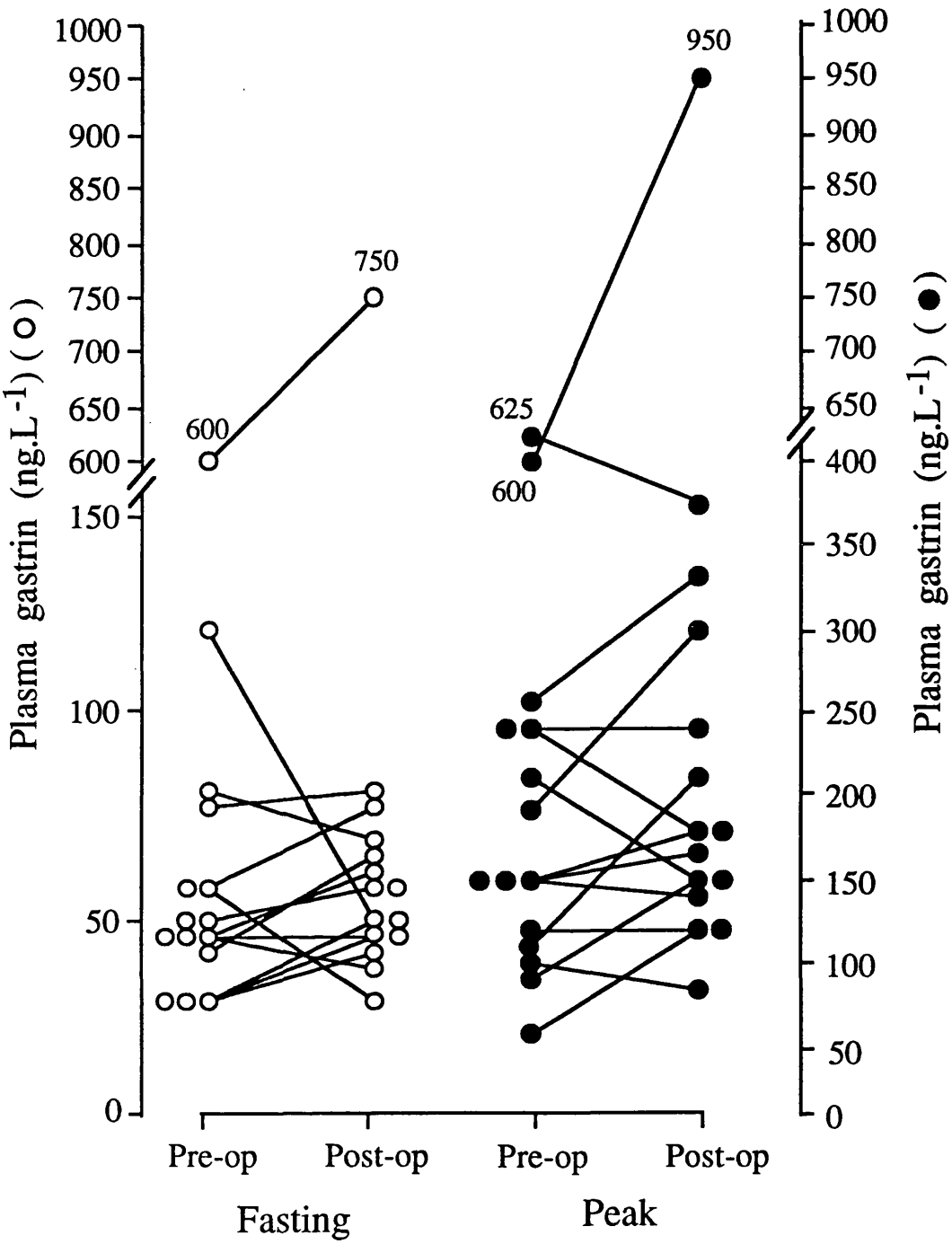


Figure 10.4

Pre- and postoperative plasma gastrin concentrations ($ng.L^{-1}$) in colorectal tumour patients with presumed curative resection. Note breaks in Y-axis.

stage and differentiation (Table 10.12). They had a similar median fasting plasma gastrin (60ng.L^{-1}, 50 - 97.5) to the twenty-five who were seen post-operatively (55ng.L^{-1}, 45-80; P = 0.38, Mann Whitney). Similarly their median peak plasma gastrin (240ng.L^{-1}, 137.5 - 395) did not differ significantly from that in the patients who were reassessed (175 ng.L^{-1}, 127.5 - 251.2; P = 0.52, Mann Whitney).

10.3.3 *Helicobacter pylori* Status and Gastrin

Using the [^{14}C]urea breath test and serology, both tumour and control groups were well matched for the presence of HP infection. Preoperatively twenty-five of the forty-two tumour patients (60%) and eighteen of the thirty-four controls (53%) were positive by urea breath test (χ^2 = 0.331, P > 0.5). Similarly, 57% of patients and 50% of controls were positive by serology, with circulating IgG levels greater than 15 U.ml^{-1} (see Figure 10.5 and Tables 10.5 and 10.6). Of the twenty-five tumour patients studied before and after tumour resection, twelve were HP positive before surgery but only seven were positive after surgery, the infection having been cleared or eradicated in five cases (see Discussion). When reassessed postoperatively, fasting gastrin was lower in four of the five patients and peak gastrin was lower in all five (Figure 10.6 and Table 10.7). The effect of loss of HP infection on gastrin levels in one patient is shown in Figure 10.7.

10.3.4 Parietal Cell/Intrinsic Factor Antibody Status and Gastrin

Intrinsic factor (type I) antibodies were present in three tumour patients (7.1%) and in two control patients (5%). Less specific parietal cell antibodies were not found in any tumour patients but were present in four controls (12%). One of the three tumour patients with positive antibodies had a fasting gastrin preoperatively of 600ng.L^{-1} which did not rise with the test meal. When seen postoperatively, his fasting and peak levels were 750 and 900ng.L^{-1}, respectively. Similarly, three of the six control patients with positive antibodies also had markedly elevated gastrin levels (see Table 10.7). In none of these cases was the diagnosis of pernicious anaemia known and no patient had

					preoperative					postoperative			
Case No.	**Initials**	**Age**	**Sex**		**10 mins**	**20 mins**	**30 mins**	**+/-**		**10 mins**	**20 mins**	**30 mins**	**+/-**
502088	G.A.	67	F		9	11	11	-		6	5	5	-
322234	J.A.	70	M		272	290	270	+		177	231	214	+
835887	H.B.	66	M		485	509	443	+		12	11	11	-
242117	J.B.	59	M		347	397	326	+					
936325	D.BU.	61	F		12	17	18	-		6	8	13	-
251770	I.C.	78	M		18	14	9	-					
649010	J.CO.	70	M		7	9	11	-		10	9	12	-
847494	A.C.	53	F		11	9	11	-		6	4	4	-
936921	J.C3.	46	M		6	5	5	-		0.5	0	0	-
855961	I.D.	67	F		9	9	12	-					
473785	A.F.	79	F		n/a	198	n/a	+					

Table 10.5a. (4 pages). [^{14}C]urea breath test results in tumour patients. + = positive, - = negative. n/a = no result.

					preoperative					postoperative			
Case No.	**Initials**	**Age**	**Sex**		**10 mins**	**20 mins**	**30 mins**	**+/-**		**10 mins**	**20 mins**	**30 mins**	**+/-**
454435	J.G.	78	M		147	179	197	+					
480507	E.G.	52	F		11	10	10	-		4	3	3	-
929989	H.S.	72	F		146	158	201	+					
497088	J.H.	47	M		4	3	3	-		4	3	4	-
121591	M.H.	66	F		406	362	437	+		246	252	249	+
516043	A.J.	79	M		99	172	194	+		17	23	26	-
738266	J.K.	67	M		183	217	229	+					
62502	A.L.	93	F		1	1	n/a	-		0	0	0	-
222730	P.L.	75	M		11	10	9	-		38	31	19	-
850663	I.M.	64	F		n/a	345	360	+					
542878	I.McC.	79	M		197	202	198	+					
657891	J.McD.	76	F		128	215	n/a	+					

Table 10.5a contd. (page 2 of 4). [^{14}C]urea breath test results in tumour patients. + = positive, - = negative. n/a = no result.

					preoperative					postoperative			
Case No.	**Initials**	**Age**	**Sex**		**10 mins**	**20 mins**	**30 mins**	**+/-**		**10 mins**	**20 mins**	**30 mins**	**+/-**
402383	M.McD.	66	F		272	231	n/a	+					
165739	W.McI.	79	M		418	433	393	+		12	10	11	-
394247	G.McK.	71	M		122	159	159	+		223	261	256	+
443246	T.McL.	58	M		n/a	n/a	n/a	- *		9	6	6	-
474018	R.McN.	75	M		175	202	205	+					
928284	E.McN.	54	F		274	279	260	+		262	301	281	+
564327	N.McR.	54	M		7	7	7	-					
709277	M.McR.	80	F		290	329	343	+					
810870	M.M.	56	M		66	102	166	+					
410814	G.N.	68	M		15	14	11	-		4	3	3	-
636073	M.P.	65	F		98	132	150	+		4	3	4	-

Table 10.5a contd. (page 3 of 4). [^{14}C]urea breath test results in tumour patients.
+ = positive, - = negative. n/a = no result. *normal antral biopsy

					preoperative					postoperative			
Case No.	**Initials**	**Age**	**Sex**		**10 mins**	**20 mins**	**30 mins**	**+/**		**10 mins**	**20 mins**	**30 mins**	**+/-**
817655	I.R.	72	M		15	16	17	-					
807750	M.R.	75	F		338	254	211	+					
562802	A.S.	79	F		248	343	352	+		72	111	157	+
922552	J.S.	80	M		9	13	18	-		10	13	17	-
372926	B.T.	44	M		195	201	228	+		186	218	203	+
632272	T.T.	87	M		185	192	213	+		9	7	6	-
850891	C.W	71	M		386	385	369	+		238	244	256	+
440457	T.W.	63	M		3	3	3	-		3	3	4	-

Table 10.5a contd. (page 4 of 4). [^{14}C]urea breath test results in tumour patients.
+ = positive, - = negative. n/a = no result.

Case No.	Initials	Age	Sex		10 mins	20 mins	30 mins	+/-
338439	D.A.	66	M		25	21	19	-
328904	W.BR.	75	M		340	338	288	+
349280	A.C2	64	F		238	315	388	+
543298	A.CH	59	M		225	260	291	+
857008	H.C.	52	M		205	236	315	+
502346	M.C2.	79	F		12	11	n/a	-
436192	T.C.	71	M		n/a	320	n/a	+
673591	J.C.	42	M		5	5	5	-
99707	J.C2.	75	M		333	407	395	+
298909	M.C3.	68	F		9	9	9	-
66109	J.DOC.	7I	M		6	7	8	-
485107	J.DU.	88	F		n/a	4	4	-
570157	E.E.	46	F		234	248	145	+
922689	J.F.	56	M		387	423	432	+

Table 10.5b. (2 pages). [^{14}C]urea breath test results in control patients. + = positive, - = negative. n/a = no result.

Case No.	Initials	Age	Sex		10 mins	20 mins	30 mins	+/-
806016	O.I.	65	F		18	16	n/a	-
n/a	P.K.	60	M		52	66	84	+
650775	J.M.	36	M		2	1	2	-
836047	G.M.	74	M		18	21	22	-
593439	L.M.	85	M		-	-	-	+*
317466	H.McB.	76	F		5	3	1	-
426207	J.McG.	46	M		234	285	325	+
754914	S.McI.	77	F		143	180	n/a	+
933860	W.McL.	72	M		172	204	234	+
640994	P.McP.	39	M		3	4	4	-
874293	A.M.	49	M		165	203	214	+
707804	M.P.	87	F		5	5	5	-
468860	S.R.	64	F		14	12	13	-
613277	H.S2.	74	F		20	9	10	-
741481	M.T.	80	F		169	n/a	172	+
504402	B.T2	64	F		7	7	7	-
643689	W.T.	66	M		189	227	189	+

Table 10.5b contd. (page 2 of 2). [^{14}C]urea breath test results in control patients.
+ = positive, - = negative. n/a = no result.

Case No.	Initials	Age	Sex	Preop. IgG titre	+/-	Postop. IgG titre	+/-
502088	G.A.	68	F	<6.25	-	8	-
322234	J.A.	70	M	51	+	14	-
835887	H.B.	66	M	19	+	80	+
242117	J.B.	59	M	9	-		
936325	D.BU.	61	F	<6.25	-	<6.25	-
251770	I.C.	78	M	<6.25	-		
649010	J.CO.	71	M	28	+	18	+
847494	A.C.	53	F	<6.25	-	22	+
936921	J.C3.	46	M	37	+	17	+
855961	I.D.	67	F	<6.25	-		
473785	A.F.	79	F	100	+		
454435	J.G.	78	M	7	-		
480507	E.G.	52	F	27	+	<6.25	-
497088	J.H.	47	M	<6.25	-	<6.25	-
121591	M.H.	66	F	117	+	35	+
516043	A.J.	79	M	63	+	38	+
738266	J.K.	69	M	14	+		
62502	A.L.	93	F	11	-	<6.25	-
222730	P.L.	75	M	54	-	89	+
850663	I.M.	64	F	56	+		
542878	I.McC.	79	M	18	+		
657891	J.McD.	76	F	<6.25	-		
402383	M.McD.	68	F	25	+		
165739	W.McI.	79	M	<6.25	-	<6.25	-
394247	G.McK.	71	M	80	+	10	-
443246	T.McL.	57	M	10	-	<6.25	-
474018	R.McN.	75	M	9	-		
928284	E.McN.	55	F	15	+	34	+
564327	N.McR.	54	M	<6.25	-		
709277	M.McR.	80	F	<6.25	-		
810870	M.M.	56	M	29	+		
410814	G.N.	69	M	22	+	<6.25	-
636073	M.P.	65	F	20	+	<6.25	-
817655	I.R.	73	M	<6.25	-		
807750	M.R.	75	F	100	+		
929989	H.S.	73	F	36	+		
562802	A.S.	79	F	63	+	37	+
922552	J.S.	81	M	11	-	77	+
372926	B.T.	44	M	24	+	<6.25	-
632272	T.T.	87	M	21	+	23	+
850891	C.W	70	M	18	+	<6.25	-
440457	T.W.	63	M	16	+	13	-

Table 10.6a. *H. Pylori* IgG titres ($U.ml^{-1}$) in tumour patients. + positive, - negative.

Case No.	Initials	Age	Sex	Preop. IgG titre	+/-
338439	D.A.	66	M	11	-
328904	W.BR.	75	M	91	+
349280	A.C2	65	F	51	+
543298	A.CH	59	M	22	+
857008	H.C.	52	M	70	+
502346	M.C2.	79	F	10	-
436192	T.C.	71	M	>200	+
673591	J.C.	42	M	<6.25	-
99707	J.C2.	75	M	49	+
298909	M.C3.	68	F	<6.25	-
66109	J.DOC.	71	M	56	+
485107	J.DU.	88	F	<6.25	-
570157	E.E.	46	F	>200	+
922689	J.F.	56	M	17	+
850006	J.F2.	73	M	129	+
532357	C.G.	63	F	43	+
698715	H.H.	72	M	105	+
806016	O.I.	65	F	<6.25	-
n/a	P.K.	60	M	17	+
650775	J.M.	36	M	<6.25	-
836047	G.M.	74	M	<6.25	-
593439	L.M.	85	M	12	-
317466	H.McB.	76	F	<6.25	-
426207	J.McG.	46	M	>200	+
754914	S.McI.	77	F	17	+
933860	W.McL.	72	M	11	-
640994	P.McP.	39	M	<6.25	-
874293	A.M.	49	M	<6.25	-
707804	M.P.	87	F	<6.25	-
468860	S.R.	64	F	<6.25	-
613277	H.S2.	74	F	21	+
741481	M.T.	80	F	>200	+
504402	B.T2	64	F	<6.25	-
643689	W.T.	66	M	11	-

Table 10.6b. *H. Pylori* IgG titres ($U.ml^{-1}$) in control patients. + positive, - negative.

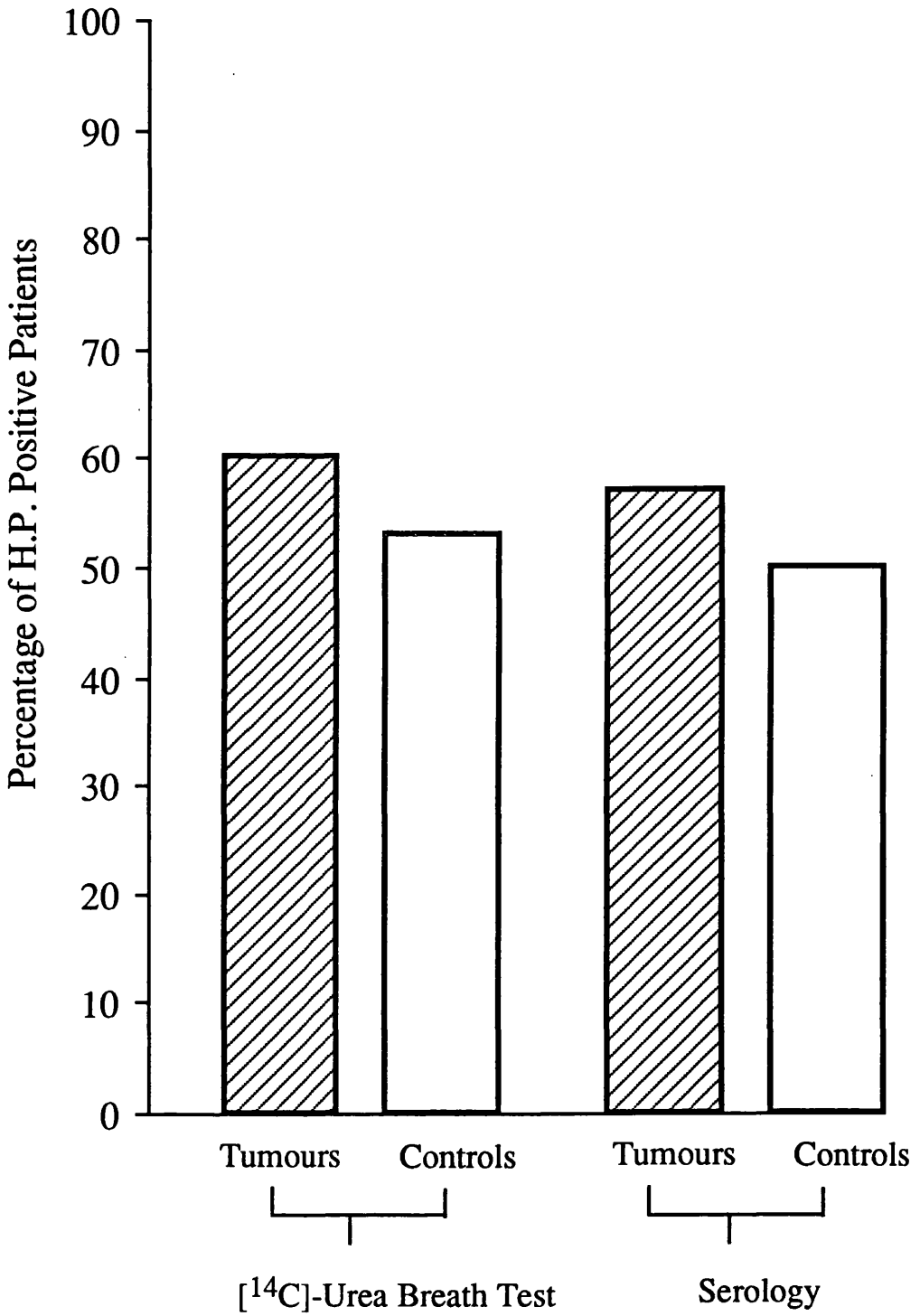


Figure 10.5

Helicobacter pylori status of study patients using both [^{14}C]-urea breath test and serology.

				Fasting			Peak	
Case No.	**Initials**	**Age**	**Sex**	**Preoperative**	**Postoperative**		**Preoperative**	**Postoperative**
636073	M.P.	65	F	140	120		480	260
516043	A.J.	79	M	75	80		200	160
632272	T.T.	87	M	80	45		150	70
165739	W.McI	79	M	55	40		150	100
835887	H.B.	66	M	70	60		200	140

Table 10.7. Plasma gastrin concentrations ($ng.L^{-1}$) in five tumour patients with perioperative loss of *Helicobacter Pylori* infection.

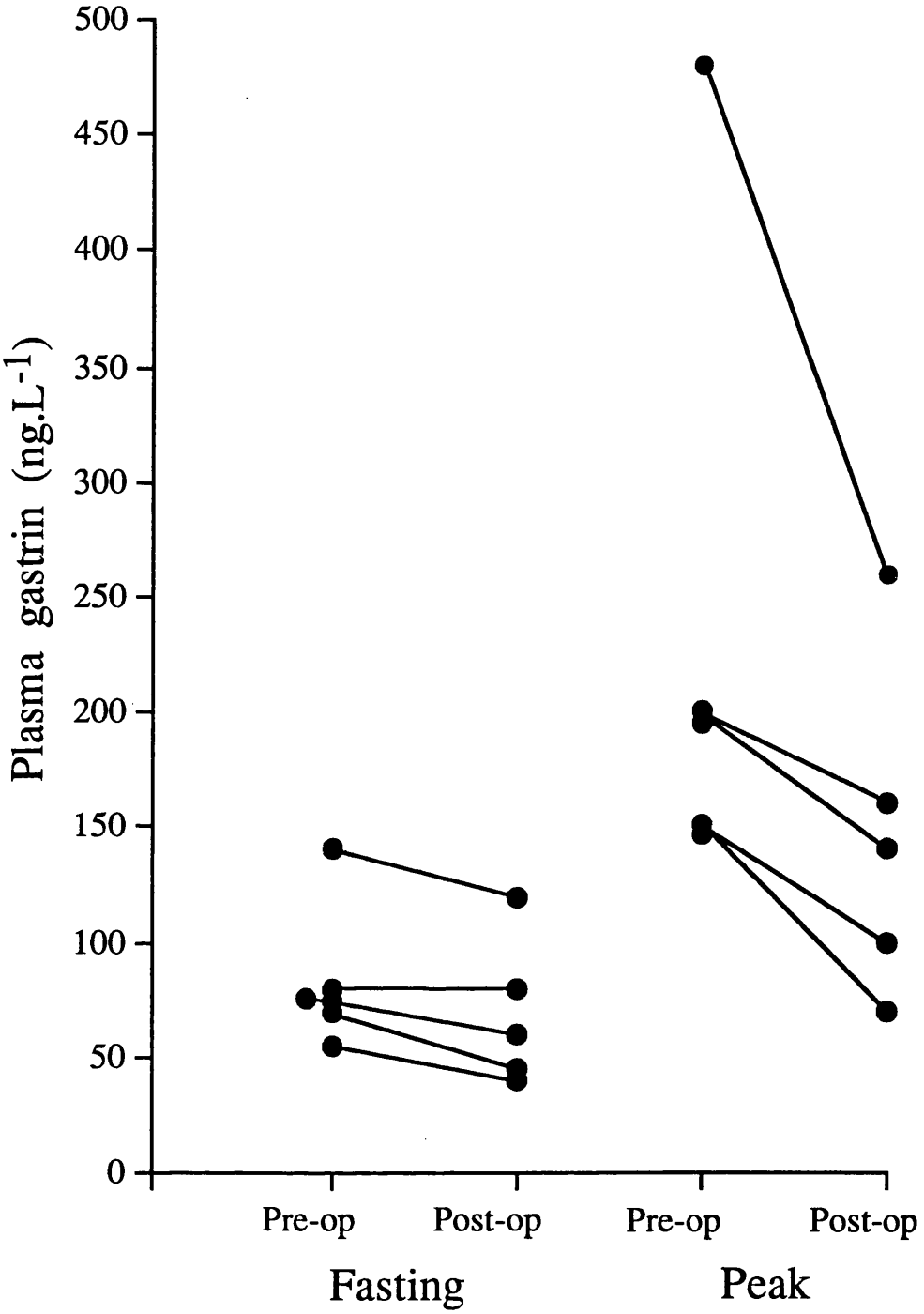


Figure 10.6

Fasting and peak plasma gastrin concentrations pre- and postoperatively in five patients with perioperative loss of *Helicobacter pylori* infection.

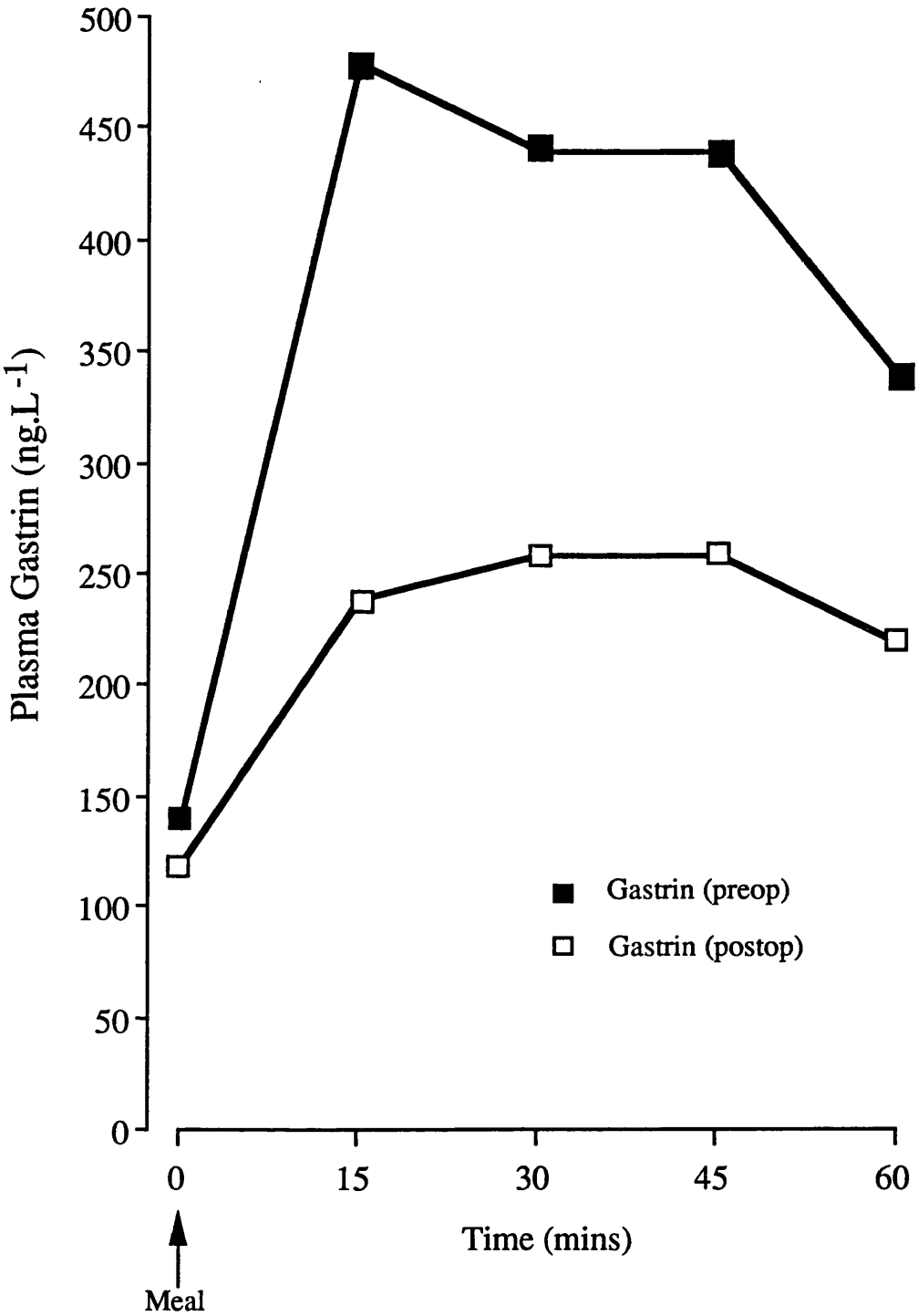


Figure 10.7

Meal-stimulated plasma gastrin concentrations ($ng.L^{-1}$) pre- and postoperatively in one patient (M.P.) with perioperative loss of HP infection.

macrocytosis, low serum vitamin B_{12} levels or other evidence of this condition. Autoantibody results are given in Table 10.9.

10.3.5 Gastrin Levels and Tumour Stage

Two patients had large benign adenomas with severe dysplasia and only one patient was classified as having a Dukes' stage A lesion. Details of the tumour histology in these patients is given in Table 10.2. There were no significant differences in either fasting (P = 0.07) or peak (P = 0.18, Kruskal-Wallis) plasma gastrin levels when analysed according to tumour stage. Median gastrin concentrations for patients with Dukes' B, C and D carcinomas are given in Table 10.10.

10.3.6 Gastrin Levels and Tumour Site

Of the forty-two tumour patients there were eight caecal carcinomas, nine descending colon or sigmoid carcinomas and twenty-five rectal tumours (twenty-three carcinomas and two large, severely dysplastic adenomas). Results of analysis of gastrin levels according to tumour site are summarised in Table 10.11. There were no significant differences among the subgroups with respect to either fasting (P = 0.62) or peak gastrin levels (P = 0.47, Kruskal-Wallis).

10.4 Discussion

The present study demonstrates that colorectal cancer patients have similar gastrin levels (fasting and peak) to control patients when studied in a carefully controlled manner. In addition, plasma gastrin did not fall following apparently curative tumour resection. The finding of no difference in gastrin levels between tumour and control patients is in keeping with that of Kikendall *et al* (1992) and several others (Suzuki *et al* 1988; Kaufmann and Ottenjann 1991; Yapp *et al* 1992; Scotté *et al* 1992). The findings differ, however, from those of previous studies which found tumour patients to have

Case No.	Initials	Age	Sex	Tumour/ Control	IF Ab	PC Ab	Fasting gastrin ($ng.L^{-1}$)	Peak gastrin ($ng.L^{-1}$)
222730	P.L.	75	M	T	+	-	600	600
810870	M.M.	56	M	T	+	-	40	165
850891	C.W.	70	M	T	+	-	80	190
673591	J.C.	75	M	C	+	-	55	340
532357	C.G.	63	F	C	+	-	325	900
502346	M.C2.	79	F	C	-	+	900	2250
468860	S.R.	64	F	C	-	+	70	140
754914	S.McI.	77	F	C	-	+	230	375
543298	A.CH.	59	M	C	-	+	50	75

Table 10.8. Plasma gastrin concentrations in patients with positive intrinsic factor (IF) or parietal cell (PC) autoantibodies. T = tumour patient, C = control patient.

Case No.	Initials	Age	Sex	Parietal Cell Antibodies (+/-)	Intrinsic Factor Antibodies (+/-)
502088	G.A.	68	F	-	-
322234	J.A.	70	M	-	-
835887	H.B.	66	M	-	-
242117	J.B.	59	M	-	-
936325	D.BU.	61	F	-	-
251770	I.C.	78	M	-	-
649010	J.CO.	71	M	-	-
847494	A.C.	53	F	-	-
936921	J.C3.	46	M	-	-
855961	I.D.	67	F	-	-
473785	A.F.	79	F	-	+/-
454435	J.G.	78	M	-	-
480507	E.G.	52	F	-	-
497088	J.H.	47	M	-	-
121591	M.H.	66	F	-	-
516043	A.J.	79	M	-	-
738266	J.K.	69	M	-	-
62502	A.L.	93	F	-	-
222730	P.L.	75	M	-	+
850663	I.M.	64	F	-	-
542878	I.McC.	79	M	-	-
657891	J.McD.	76	F	-	-
402383	M.McD.	68	F	-	-
165739	W.McI.	79	M	-	-
394247	G.McK.	71	M	-	-
443246	T.McL.	57	M	-	-
474018	R.McN.	75	M	-	-
928284	E.McN.	55	F	-	-
564327	N.McR.	54	M	-	-
709277	M.McR.	80	F	-	-
810870	M.M.	56	M	-	+
410814	G.N.	69	M	-	-
636073	M.P.	65	F	-	-
817655	I.R.	73	M	-	-
807750	M.R.	75	F	-	-
929989	H.S.	73	F	-	-
562802	A.S.	79	F	-	-
922552	J.S.	81	M	-	-
372926	B.T.	44	M	-	-
632272	T.T.	87	M	-	-
850891	C.W	70	M	-	+
440457	T.W.	63	M	-	-

Table 10.9a. Gastric autoantibody status of tumour patients. + positive, - negative.

Case No.	Initials	Age	Sex	Parietal Cell Antibodies (+/-)	Intrinsic Factor Antibodies (+/-)
338439	D.A.	66	M	-	-
328904	W.BR.	75	M	-	-
349280	A.C2	65	F	-	-
543298	A.CH	59	M	+	-
857008	H.C.	52	M	-	-
502346	M.C2.	79	F	+	-
436192	T.C.	71	M	-	-
673591	J.C.	42	M	-	-
99707	J.C2.	75	M	-	+
298909	M.C3.	68	F	-	-
66109	J.DOC.	71	M	-	-
485107	J.DU.	88	F	-	-
570157	E.E.	46	F	-	-
922689	J.F.	56	M	-	-
850006	J.F2.	73	M	-	-
532357	C.G.	63	F	-	+
698715	H.H.	72	M	-	-
806016	O.I.	65	F	-	-
n/a	P.K.	60	M	-	-
650775	J.M.	36	M	-	-
836047	G.M.	74	M	-	-
593439	L.M.	85	M	-	-
317466	H.McB.	76	F	-	-
426207	J.McG.	46	M	-	-
754914	S.McI.	77	F	+	-
933860	W.McL.	72	M	-	-
640994	P.McP.	39	M	-	-
874293	A.M.	49	M	-	-
707804	M.P.	87	F	-	-
468860	S.R.	64	F	+	-
613277	H.S2.	74	F	-	-
741481	M.T.	80	F	-	-
504402	B.T2	64	F	-	-
643689	W.T.	66	M	-	-

Table 10.9b. Gastric autoantibody status of control patients. + positive, - negative.

	Fasting			Peak	
	Median†	**IQ range**		**Median†**	**IQ range**
caecum (n=8)	62.5	35 - 131.2		175	67.5 - 468.8
sigmoid/descending colon (n=9)	50	40 - 77.5		150	115 - 468.8
rectum (n=25)	55	45 - 105		210	150 - 326.2

Table 10.10. Fasting and peak meal-stimulated plasma gastrin concentrations ($ng.L^{-1}$) according to tumour site. IQ = interquartile.

	Fasting			Peak	
	Median†	**IQ range**		**Median†**	**IQ range**
Dukes B (n=12)	77.5	51.2-116.3		220	127.5-468.8
Dukes C (n=16)	50	45-73.75		150	101.2-232.5
Dukes D (n=11)	70	50-120		250	146.3-363.7

Table 10.11. Fasting and peak meal-stimulated plasma gastrin concentrations ($ng.L^{-1}$) according to tumour stage. IQ = interquartile.

	25 patients reassessed	17 patients not reasssessed
Patients		
males	16	9
females	9	8
median age (range)	67 (44-93)	72 (54-80)
Site		
caecum	5	3
desc./sigmoid colon	7	3
rectum	13	11
Stage		
adenoma	0	2
A	1	0
B	11	1
C	9	6
D	4	8
Median Gastrin		
fasting (IQ range)	55 (45-80)	60 (50-97.5)
peak (IQ range)	175 (127.5-251.2)	240 (137.5-395)

Table 10.12. Details of colorectal tumour patients reassessed and not reassessed postoperatively.

higher gastrin levels than controls (Smith *et al* 1989; Wong *et al* 1991; Seitz *et al* 1992; Charnley *et al* 1992).

Several reasons may account for these conflicting reports. The first explanation is that different methods of statistical analysis have been used to interpret gastrin concentrations in previous studies. In the original study of Smith *et al* (1989), *mean* fasting gastrin levels were elevated eight-fold compared to controls but this was because a subgroup (8/20) of tumour patients had very high gastrin levels. Other authors have also found elevated gastrin levels to exist in only a minority of patients although the aetiology of these high levels was unexplained (Wong *et al* 1991; Yapp *et al* 1992; Kikendall *et al* 1992). In these studies, therefore, gastrin levels were most unlikely to have been normally distributed making the use of means, standard deviations and parametric statistical tests inappropriate. When these high values (or "outliers") have been excluded from analysis there has been no significant difference in gastrin levels between tumour patients and controls (Yapp *et al* 1992; Kikendall *et al* 1992). Analysis of results in this study showed that the distribution of both fasting and peak gastrin levels in both groups deviated significantly from normality (Wilk-Shapiro, $P < 0.01$). In keeping with the findings of Kikendall *et al* (1992), logarithmic transformation failed to normalise the results and so non-parametric tests were used for analysis of gastrins. Inspection of the individual gastrin levels in Figure 10.3 shows that very high fasting gastrin levels (>200ng.L^{-1}) occurred in only one tumour patient (with positive intrinsic factor antibodies) but in five control patients of whom three had positive gastric autoantibodies. Expressing results as medians rather than means, combined with non-parametric statistical analysis, is more appropriate.

The second reason is failure to control for factors which may have raised gastrin in some patients. Thus, patients taking acid-suppressing drugs (H_2-antagonists, proton pump inhibitors) were excluded from the study as were those with hypercalcaemia or renal impairment. Another confounding factor in previous studies may have been failure to control for the presence of pernicious anaemia. Although patients with known pernicious anaemia have been excluded from most of the earlier studies the condition is relatively common and often asymptomatic in this age group.

This is the first study to try and control for the presence of pernicious anaemia. Although not definitive, the presence of positive gastric autoantibodies in combination with very high gastrin levels is highly suggestive of this diagnosis. Positive autoantibodies were found in three tumour patients and in six control patients (Table 10.8) and these could readily explain all the very high gastrin concentrations seen in the study with the exception of one patient in the control group with a fasting gastrin of $750ng.L^{-1}$.

A further potential source of error in previous studies may have arisen because of failure to appreciate the possible effects on gastrin levels of bowel preparation given prior to colonoscopy or surgery. Several studies measured gastrin concentrations immediately prior to these procedures *i.e.* after bowel preparation (Suzuki *et al* 1988; Smith *et al* 1989; Seitz *et al* 1989; Charnley *et al* 1992). Many aspects of gastrointestinal function, including motility, are under neuroendocrine regulation (Sakomoto *et al* 1987) and are clearly affected by use of powerful laxatives or intestinal lavage. While the effects of bowel preparation on gastrin levels *per se* are unknown it is a cause for concern, especially as postoperative gastrin levels were measured without the effects of such bowel preparation making interpretation of differences in hormone levels difficult.

A final reason for the conflicting results of previous studies may be failure to control for the prevalence of HP in the different study groups. This is potentially very important as the infection causes significant hypergastrinaemia (McColl *et al* 1989; Levi *et al* 1989). Using the [^{14}C]urea breath test and serology the prevalence of the infection was similar in tumour patients and controls. One can thus exclude differences in the relative prevalence of HP infection between the two groups as a confounding factor in this study. In contrast, no other study of gastrin levels in colorectal tumour patients has controlled for the presence of this infection. It is therefore possible that previously reported hypergastrinaemia in tumour patients may have been due, at least in part, to different proportions of HP positive subjects in the tumour and control groups. Very recently preliminary results from another group have supported this finding in that only

HP-positive colorectal cancer patients had higher gastrin levels than the control group (Meier *et al* 1994).

The second finding of this study is that no fall in gastrin was observed after apparently curative resection. While this is in agreement with the results of Scotté *et al* (1992) and others (Kikendall *et al* 1992), it differs from the findings of several authors who have reported a fall in gastrin levels postoperatively (Wong *et al* 1991; Seitz *et al* 1992; Charnley *et al* 1992). Although the possible effect of bowel preparation noted above may have contributed, it cannot fully explain the apparent fall in gastrin as Wong *et al* were careful to avoid bowel preparation in their study (Wong *et al* 1991). Perhaps more importantly, none of the studies which noted postoperative reductions in gastrin controlled for the presence of HP infection and it is possible that clearance or eradication of the infection in the perioperative period may have occurred in some patients, as noted in the present study. In this study the infection had been cleared or eradicated in five of the twenty-five patients who were restudied postoperatively and this was associated with falls in both fasting and meal-stimulated gastrin levels. When these five patients were excluded from analysis there was no difference in median gastrin levels before and after surgery. From Figure 10.4 it can be seen that, of the fifteen patients, reductions in fasting gastrin occurred in only four. Likewise peak gastrin levels fell in only five cases, whereas they rose in eight. Furthermore the falls were minor and unlikely to be of biological significance. It seems unlikely, therefore, that curative tumour resection results in a lowering of gastrin concentrations by removal of the source of gastrin (or a peptide which stimulates gastrin release). If gastrin is an autocrine growth factor in this condition it is probably effective at very low tissue concentrations without affecting circulating levels.

Alternatively, the reductions in gastrin seen postoperatively in five (20%) of our patients can readily be explained by clearance or eradication of HP infection in each of them. The reason for the loss of the infection is unclear but may have resulted from the perioperative intravenous antibiotics given routinely to patients undergoing colorectal surgery. All five patients received three doses of metronidazole (effective against HP) and cefotaxime and it is possible that this contributed to eradication of the

infection. In addition, three of the five had required postoperative antibiotics for either chest or urinary tract infections and this may also have been important in eradicating the organism. Figures 10.6 and 10.7 demonstrate the effect of perioperative loss of HP infection on meal-stimulated plasma gastrin concentrations in this study. It is therefore possible that previously reported postoperative reductions in gastrin (Wong *et al* 1991; Seitz *et al* 1992; Charnley *et al* 1992) may have resulted, not from tumour resection *per se*, but from coincidental eradication of HP infection in the perioperative period.

For reasons explained above (see Results), only twenty-five of the forty-two colorectal tumour patients were reassessed postoperatively. The seventeen who were not restudied were similar to these twenty-five with respect to patient characteristics and tumour details (Table 10.12). In addition, their fasting and peak plasma gastrin levels were similar making it unlikely that those seen again postoperatively differed in any way from the overall group.

There was also a wide range in the timing of postoperative reassessment in the study. Rather than seeing the patients at one fixed time point postoperatively, and at different stages in their recovery, the principal concern was to reassess patients once they had fully recovered from the effects of surgery and had returned to as near a normal lifestyle as possible. In this elderly population, recovery rates vary considerably. Furthermore, there is no evidence that gastrin levels in an individual change significantly over a six month period, unless possibly due to acquisition of HP infection and the study controlled for such a possibility. Analysis of the data shows no correlation between timing of postoperative reassessment and either fasting (Spearman's rank correlation coefficient, $\rho_S = 0.187$; $P > 0.1$) or peak plasma gastrin ($\rho_S = 0.246$; $P > 0.1$). For these reasons it is likely that the range in timing of postoperative reassessment had no significant influence on the results and the study design used was appropriate in this patient population.

In this present study, which has controlled for factors which may elevate circulating gastrin levels, normal levels were found in colorectal tumour patients with no fall following apparently curative tumour resection. The findings therefore provide no support for colorectal tumours directly or indirectly *causing* elevated circulating

gastrin concentrations and have subsequently been corroborated by the results of Rogy *et al* (1994). While the study only involved patients with sporadic tumours, there is little reason to suspect that the situation is different in patients with familial adenomatous polyposis and this has subsequently been confirmed (Svendson *et al* 1994).

The finding of normal levels, however, does not exclude gastrin having a trophic effect on colorectal tumours as the hormone can exert such effects at physiological levels (Johnson 1987). In addition the study does not exclude the local production of gastrin by colorectal tumours with consequent autocrine or paracrine effects on tumour growth. Recent studies demonstrating gastrin gene expression in some colonic cancer cell lines (Baldwin 1992) and progastrin-derived peptides in human tumours (Kochman *et al* 1992; Nemeth *et al* 1993) do support the concept of a role for gastrin as an autocrine growth factor in this condition. The next chapter addresses whether measurable amounts of gastrin and its precursors are present in the tumours of this group of colorectal tumour patients.

CHAPTER 11

GASTRIN AND GASTRIN PRECURSORS IN HUMAN COLORECTAL NEOPLASMS

11.1 INTRODUCTION

As discussed in Chapter 7, recent research has addressed the possible involvement of gastrin as an autocrine or paracrine growth factor for colorectal cancer cells. If this proves to be the major effect of gastrin in this disease then one would not expect circulating gastrin levels to differ between tumour patients and healthy people because autocrine growth factors may have important effects on tumour growth even at very low levels of expression. Indeed, the results of the previous chapter would seem to indicate that plasma gastrin concentrations are not higher in colorectal tumour patients.

The aim of this study was to determine whether human colorectal tumours contained measurable amounts of either bioactive, carboxyamidated gastrins or their precursors, namely progastrin and glycine-extended gastrins.

11.2 METHODS

11.2.1 Patients and Tumours

Samples of tumour and macroscopically normal mucosa were taken from patients with colorectal cancer at surgical resection. Of the forty-two patients who took part in the study of plasma levels (Chapter 10), tissue samples were available in thirty. Eighteen tumour and mucosal samples were obtained from other unselected patients undergoing elective surgery for sporadic colorectal carcinoma. Patient details are given in Table 11.1.

Immediately on removal from the patient resection specimens were opened along the antimesenteric border and washed thoroughly in ice-cold Ringer Lactate solution to remove faeces and blood clot. Small pieces (approximately 0.5-1.0g) were excised from the tumour with care being taken to avoid areas of obvious necrosis, placed in plastic containers, snap frozen in liquid nitrogen and then stored at -70°C until required. Samples of macroscopically normal colonic mucosa were taken from an area as far removed from the tumour as possible (5-15cm) and stored as above.

Case No.	Initials	Age	Sex	Tumour site	Histology	Differentiation	Dukes stage	Site of metastases
738266	J.K.	67	M	rectum	adenocarcinoma	moderate	C	
632272	T.T.	87	M	sigmoid	adenocarcinoma	moderate	B	
480507	E.G.	52	F	caecum	adenocarcinoma	moderate	C	
62502	A.L.	93	F	rectum	adenocarcinoma	moderate	C	
537146	C.C.	65	M	caecum	adenocarcinoma	poor	C	
154269	E.R.	57	F	caecum	adenocarcinoma	poor	C	
511698	L.H.	58	M	rectum	adenocarcinoma	moderate	C	
854330	M.W.	74	F	caecum	adenocarcinoma	moderate	C	
810870	M.M.	56	M	rectum	adenocarcinoma	moderate	D	liver
928284	E.MCN.	54	F	rectum	adenocarcinoma	moderate	D	liver
882211	P.S.	56	F	sigmoid	adenocarcinoma	poor	C	
222730	P.L.	75	M	rectum	adenocarcinoma	moderate	B	
922552	J.S.	80	M	rectosigmoid	adenocarcinoma	moderate	C	
402383	M.MCD.	66	F	rectum	adenocarcinoma	moderate	D	liver
932616	M.MCL	48	M	rectosigmoid	adenocarcinoma	moderate	D	liver

Table 11.1. Clinical and tumour details of patients.

11.2.2 Extraction of Peptides

While still frozen, tissue samples were split into two approximately equal pieces, quickly weighed and labelled Sample A (for assay without trypsin treatment) and Sample B (for assay following trypsin treatment). Sample A was added to 1ml of boiling phosphate-buffered saline (PBS), finely minced with a scalpel and boiled for ten minutes to inactivate tissue enzymes. After being left to stand for one hour at 37°C, the sample was centrifuged at 10,000G for 30 minutes, the supernatant decanted and boiled again as above. The final supernatant was made up to a total volume of 2ml with PBS.

Sample B was treated in the same way except that it was incubated with 10mg trypsin beads for one hour at 37°C after initial boiling. This form of trypsin (TPCK-treated insoluble enzyme, Sigma Chemical Co., Poole, U.K.) is highly pure and easily removed by centrifugation. Final supernatants of all samples were saved at -70°C until analysed. This method has been widely used and gives good recovery of progastrin and its major processing products.

11.2.3 Radioimmunoassay of Gastrin and its Processing-intermediates

All samples were assayed using two different region-specific antisera (Figure 2.1, repeated after page 120 for clarity). Antibody R98 recognises the C-terminal of gastrins and requires the presence of the amidated C-terminal amino acid. It therefore detects all bioactive, carboxyamidated gastrins (Component I, G-34, G-17 and G-14) and both sulphated and unsulphated forms are recognised with equal potency. Cross-reactivity with CCK is < 0.005% (Ardill 1973). In standard use for measurement of plasma gastrin this antisera has a sensitivity of $3ng.L^{-1}$ and an interassay coefficient of variation of 3-6%. In the present study R98 was used at higher sensitivity with a detection limit of $300pg.L^{-1}$ (equivalent to $0.14pmol.g^{-1}$) but with a higher coefficient of interassay variation (12.6%). Higher sensitivity was achieved by increasing assay volume, decreasing the concentrations of antisera and label and increasing the incubation time. Trypsin treatment of peptide extracts cleaves progastrin to yield glycine-extended

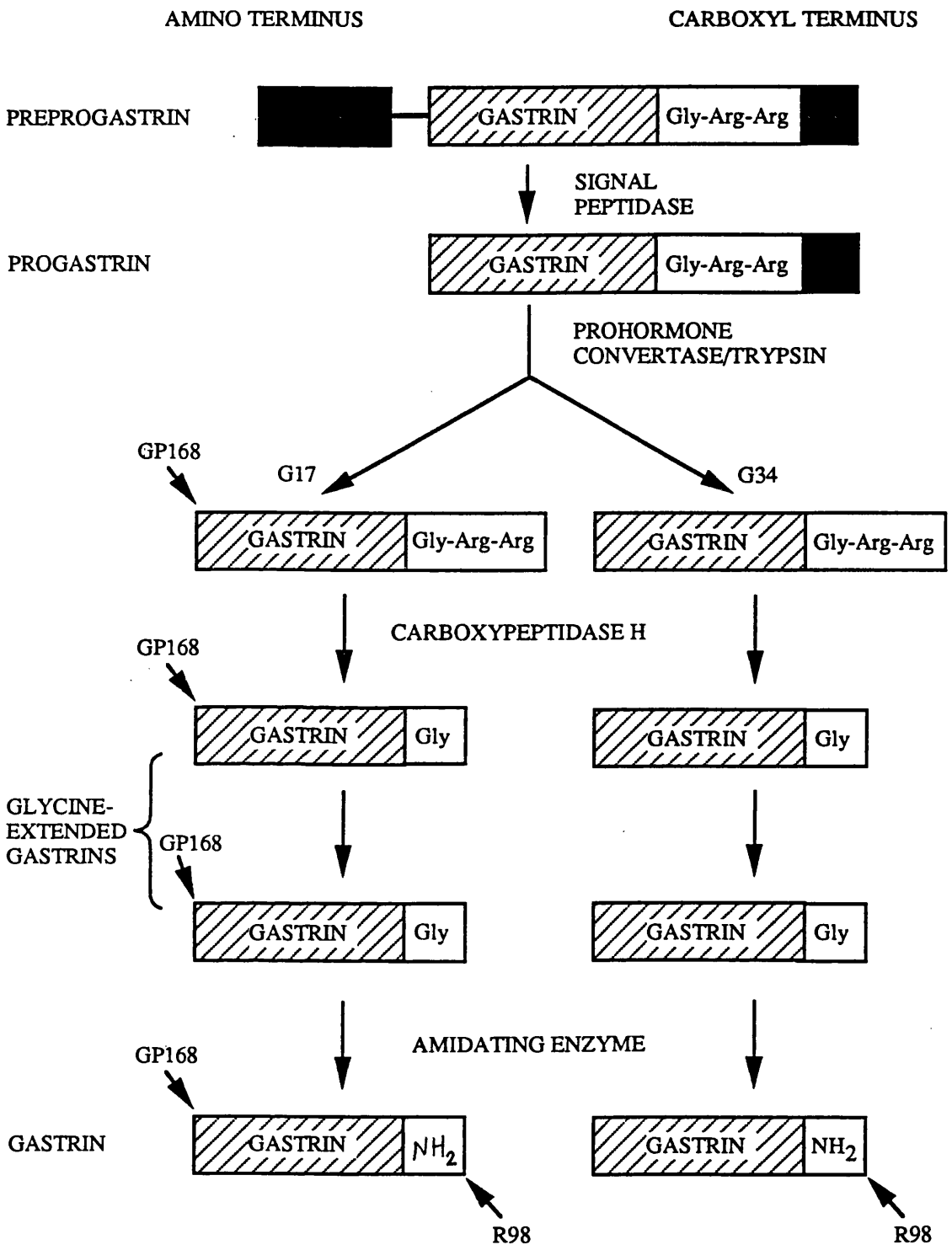


Figure 2.1

Schematic representation of gastrin processing. Arrows indicate binding sites of antigastrin antisera (R98, GP168) used in this study (see chapter 11).

gastrins and should not, therefore, affect the levels of mature, carboxyamidated gastrin measured with R98.

Antibody GP168 recognises the N-terminal region of gastrin and requires the presence of the N-terminal pyroglutamyl residue of G-17. As such it detects carboxyamidated gastrin-17, glycine-extended processing intermediates (G-17-gly and G-17-gly-arg-arg) and other short N-terminal fragments but not G-34. GP168 has a standard detection limit of approximately 7-15ng.L^{-1} but in this assay sensitivity was increased to 3ng.L^{-1} (equivalent to 1.43pmol.g^{-1}). It shows no cross-reaction with CCK (Mulholland *et al* 1993). Following trypsin digestion of preprogastrin, increased concentrations of gastrin processing intermediates should be detected by this antibody.

Peptide concentrations were measured as ng.L^{-1} and expressed as pmol.g^{-1} wet weight of tissue.

11.2.4 Statistics

Results are given as medians and ranges. Mann Whitney U test was used for comparisons between tumour tissue and disease-free mucosa, while differences in peptide content before and after trypsinisation of each tissue were assessed using Wilcoxon's matched pairs test. Correlation of peptide content in tumour tissue and matched normal mucosa was made using Spearman's rank correlation coefficient. All statistical analyses were performed using Minitab statistical software, version 8.0 (Minitab Inc., Pennsylvania, USA) on an IBM-compatible personal computer.

11.3 RESULTS

11.3.1 Radioimmunoassay

Of the samples of tumour and normal mucosa which were initially available, final results were available for fifteen cases. Initial assay with R98 was performed using standard sensitivity and a lower detection limit of 3ng.L^{-1}. In this way, no detectable gastrin-like peptides were found during analysis of samples from seventeen patients. Further

analysis was performed on twenty-five samples using an assay of greater sensitivity. In this second assay method peptide extraction was performed on one piece of tissue and the supernatant was then split into two aliquots to be assayed with or without trypsinisation. Although gastrin (1-10pg.g^{-1} wet weight) was detected in almost all samples, levels did not consistently dilute in parallel, most likely because of non-specific interference. These results were therefore excluded.

A third, modified assay was repeated on the fifteen samples still available. Each sample (tumour or disease-free mucosa) was split into two representative pieces while still frozen, as described in Methods (above). High sensitivity with a lower detection limit of 300pg.L^{-1} (i.e. 0.14pmol.g^{-1}) was achieved by increasing assay volume from 100μL to 200μL, decreasing antibody and label concentrations by 50% and lengthening incubation time to five days. In all cases the results obtained were reproducible in at least two doubling dilutions.

11.3.2 Tissue Peptide Contents

(i) Carboxyamidated gastrins

Small but measurable amounts of carboxyamidated gastrins were found in all fifteen tumour samples (median 5.4pmol.g^{-1}, range 1.0 - 141.0) and in fourteen of the fifteen samples of matched disease-free mucosa (5.0pmol.g^{-1} , 0.0 - 15.7). Results are shown in Tables 11.2 and 11.3 and Figure 11.1. There was no statistically significant difference in peptide content between tumour tissue and disease-free mucosa (P = 0.48, Mann Whitney).

As expected levels of carboxyamidated gastrins measured in disease-free mucosa were the same before (median 5.0pmol.g^{-1}, range 0.0 - 15.7) and after trypsinisation (4.8pmol.g^{-1}, 1.3 - 21.0; P = 0.57, Wilcoxon). There was, however, a small but statistically significant increase in carboxyamidated gastrins in tumours following trypsin digestion (P = 0.03). The results are depicted in Figure 11.1.

	Tumour			Normal mucosa		
	R98	GP168	GP168 post-trypsin	R98	GP168	GP168 post-trypsin
Median	11.3	12.9	43.3	10.4	17.5	20.5
Range	3.4-51.0	<1.4 - 141.0	7.4-475.0	0.0-33.0	0.0-128.0	6.8-175.0

Table 11.2. Summary of tissue content of gastrins. Results are expressed as $pmol.g^{-1}$ wet weight of tissue.

				Tumour		Normal	
Case No.	**Initials**	**Age**	**Sex**	**R98-pre**	**R98-post**	**R98-pre**	**R98-post**
738266	J.K.	67	M	6.9	13.7	5.0	6.7
632272	T.T.	87	M	3.4	5.7	4.6	4.6
480507	E.G.	52	F	9.5	11.9	9.0	6.2
62502	A.L.	93	F	5.7	3.7	1.2	1.9
537146	C.C.	65	M	4.3	6.2	2.9	5.3
154269	E.R.	57	F	4.9	5.2	10.0	4.8
511698	L.H.	58	M	5.4	7.4	2.6	2.8
854330	M.W.	74	F	2.5	3.2	2.2	2.3
810870	M.M.	56	M	1.6	2.8	2.3	4.4
928284	E.MCN.	54	F	2.9	1.5	5.0	3.4
882211	P.S.	56	F	21.4	25.2	15.7	21.0
222730	P.L.	75	M	24.3	43.3	11.0	8.1
922552	J.S.	80	M	6.7	12.4	9.0	9.5
402383	M.MCD.	66	F	5.3	1.4	0.0	1.3
932616	M.MCL	48	M	8.6	13.8	61.0	6.2

Table 11.3. Content (pmol.g^{-1} wet weight of tissue) of carboxyamidated gastrins in tumours and normal mucosa with ('pre') or without trypsin ('post') digestion.

				Tumour		Normal	
Case No.	**Initials**	**Age**	**Sex**	**GP168-pre**	**GP168-post**	**GP168-pre**	**GP168-post**
738266	J.K.	67	M	1.7	20.6	13.4	13.5
632272	T.T.	87	M	<1.4	5.7	5.8	10.3
480507	E.G.	52	F	3.8	9.1	3.8	3.9
62502	A.L.	93	F	5.3	9.4	2.7	3.2
537146	C.C.	65	M	19.0	73.8	16.7	64.3
154269	E.R.	57	F	2.8	16.3	0.0	10.0
511698	L.H.	58	M	4.4	39.4	4.9	3.9
854330	M.W.	74	F	13.0	23.8	8.3	9.8
810870	M.M.	56	M	9.8	13.5	15.4	17.9
928284	E.MCN.	54	F	22.4	26.9	5.4	3.5
882211	P.S.	56	F	6.1	9.2	19.9	9.0
222730	P.L.	75	M	17.6	77.6	15.7	17.6
922552	J.S.	80	M	22.4	38.1	8.4	7.1
402383	M.MCD.	66	F	<1.4	3.5	0.0	5.8
932616	M.MCL	48	M	141.0	226.2	5.7	83.3

Table 11.4. Content (pmol.g^{-1} wet weight of tissue) of gastrin precursors and processing intermediates.

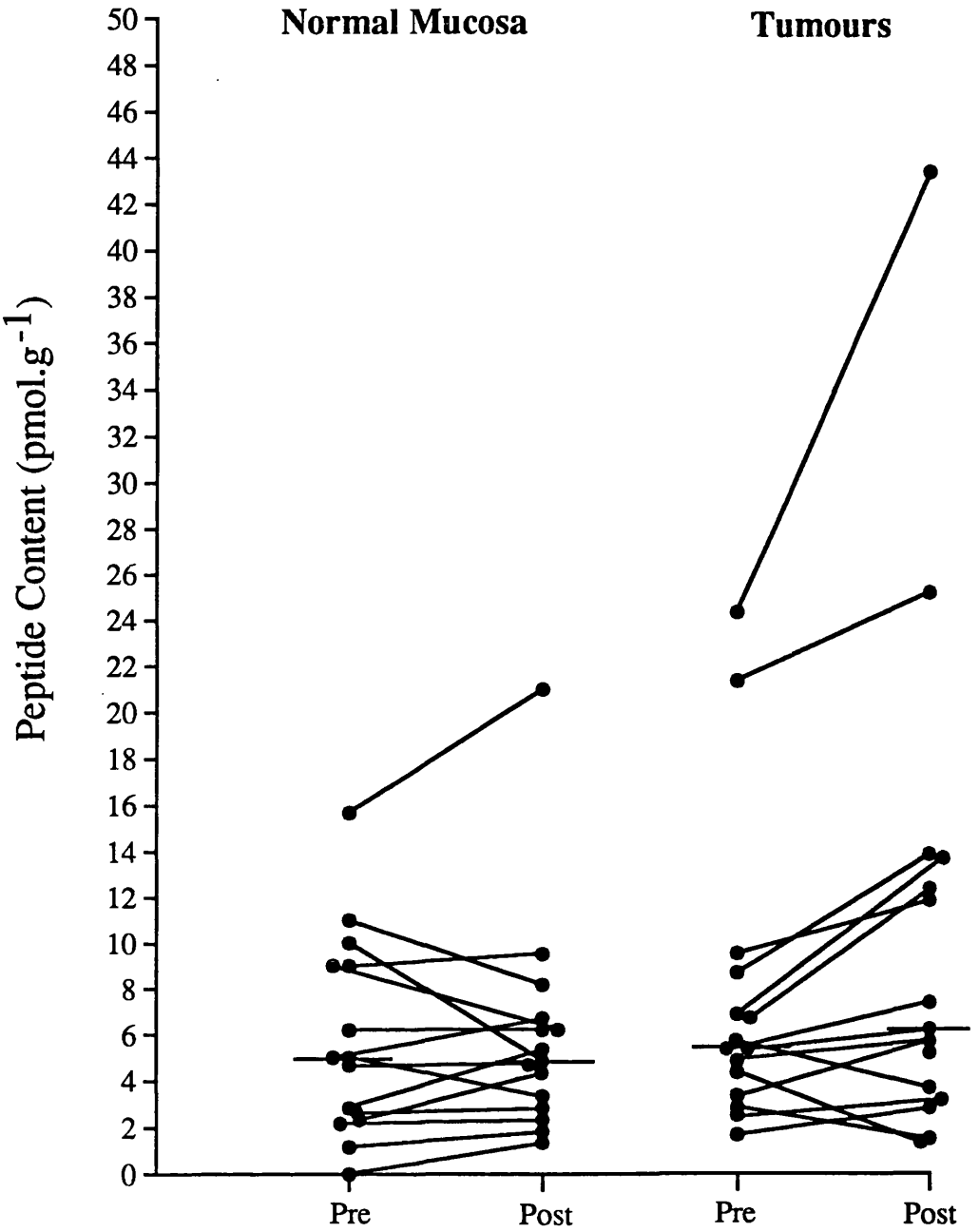


Figure 11.1

Content of carboxyamidated gastrins (pmol.g^{-1}wet weight of tissue) as measured by antisera R98.
Horizontal bars indicate median values.

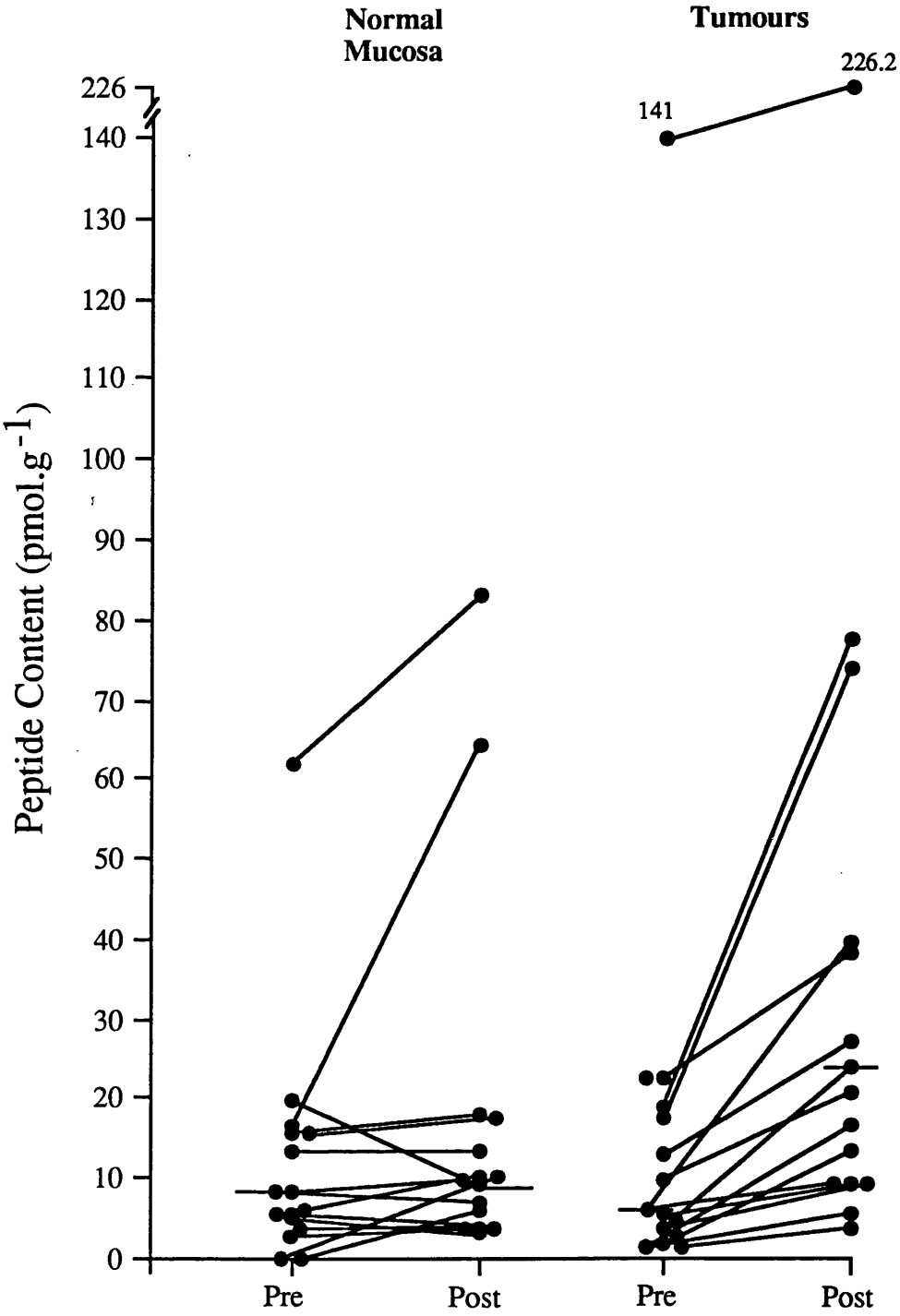


Figure 11.2

Content of gastrin processing-intermediates and precursors (pmol.g^{-1} wet weight of tissue) as measured by antisera GP168.
Horizontal bars indicate median values.

(ii) Gastrin precursors and processing-intermediates

All fifteen tumours contained measurable amounts of gastrin processing-intermediates (median 6.1pmol.g^{-1}, range 1.0 - 141.0) as did thirteen of the fifteen matched samples of disease-free mucosa (8.3pmol.g^{-1}, 0.0 - 61.0). The amounts were not significantly different in the two tissues (P = 0.92, Mann Whitney). Individual results are shown in Table 11.4 and Figure 11.2.

Following trypsin digestion there was a non-significant increase in measurable immunoreactive gastrin processing-intermediates in samples of disease-free mucosa (median 9.8pmol.g^{-1}, range 3.2 - 83.3; P = 0.09, Wilcoxon). In tumour tissues, however, trypsin treatment greatly increased the content of immunoreactive peptides (20.6pmol.g^{-1}, 3.5 - 226.2; P = 0.001, Wilcoxon). The median content of post-trypsinisation peptides (i.e. progastrin and glycine-extended gastrins) was considerably greater in tumours than in disease-free mucosa but the difference failed to reach statistical significance (P = 0.07, Mann Whitney). The content of trypsin-digested gastrin processing-intermediates measured with GP168 (Table 11.4) was significantly greater than the content of carboxyamidated gastrins in both disease-free mucosa (P = 0.026) and tumours (P = 0.001, Mann Whitney).

There was significant correlation in the content of carboxyamidated gastrins in normal mucosa and cancer tissue (Spearman's rank correlation coefficient ρ_s = 0.624, P < 0.02). Likewise, the content of gastrin-processing intermediates was also strongly correlated (ρ_s = 0.605, P = 0.02). In contrast, after trypsin digestion no significant correlation existed in the content of these precursors in the two tissues (ρ_s = 0.402, P > 0.1).

11.4 DISCUSSION

Increasing evidence supports a role for gastrin as an important autocrine/paracrine growth factor for some gastrointestinal and other tumours. Initial studies used an anti-gastrin antiserum added to the medium of colonic carcinoma cell lines and noted an

inhibition of growth as well as suggesting the presence of gastrin mRNA in cells (Hoosein *et al* 1989, 1990). Using highly sensitive PCR methods, several groups have reported gastrin gene expression in colonic carcinoma cell lines (Baldwin *et al* 1990; Baldwin and Zhang 1992; Xu *et al* 1994) and fresh colonic cancers (Monges *et al* 1993). Using flow cytometry Watson *et al* found six of twenty-eight disaggregated fresh colonic cancers to contain more than 20% gastrin-positive cells (Watson *et al* 1991). Others have also found cell line homogenates and fresh tumours to contain variable amounts of gastrin and its precursors by radioimmunoassay (Kochman *et al* 1992; Van Solinge *et al* 1993a; Nemeth *et al* 1993; Singh *et al* 1994). The levels of bioactive carboxyamidated gastrins have been very low and the biological importance of immature precursors is unclear (see below).

Gastrin-like peptides with autocrine trophic properties have also been observed in other neoplastic cell lines including gastric (Remy-Heintz *et al* 1993), pancreatic (Blackmore and Hirst 1992) and renal (Blackmore *et al* 1994). In addition, gastrin production has been noted in a significant proportion of human bronchogenic (Rehfeld *et al* 1989) and ovarian cancers (Van Solinge *et al* 1993b) and reports of 'ectopic' gastrin production by a carcinoma of oesophagus and a hepatic carcinoid tumour have been made (Nishimaki *et al* 1993; Inoue *et al* 1993). Gastrin production may thus be a feature shared by many neoplastic cells of diverse origin. Both gastrin and CCK may play important roles during gastrointestinal development (at least in rodents) with expression being 'switched off' in *normal* adult tissues (Lüttichau *et al* 1993; Marino *et al* 1994).

In this study nearly all colorectal tumours and most samples of normal mucosa were found to contain small but measurable amounts of progastrin-derived peptides. Both bioactive, carboxyamidated peptides and processing-intermediates (C-terminal glycine-extended gastrins and progastrin) were detected in both types of tissue. Overall the concentrations of carboxyamidated gastrins measured with antisera R98 were similar in both tumours and matched samples of macroscopically normal mucosa. The content of gastrin processing-intermediates detected by antisera GP168 was also similar in the two tissues and trypsin treatment greatly increased the concentration of

immunoreactive material detected by GP168 but made little difference in normal mucosa, suggesting that colorectal tumours may contain relatively greater amounts of progastrin than normal mucosa.

There was a wide range in measured concentrations (see Tables 11.3 and 11.4) among samples and this has also been observed by others (Kochman *et al* 1992; Nemeth *et al* 1993). In some reports carboxyamidated gastrins were present in much smaller concentrations (£ 1pmol.g^{-1}) than observed in this study (Kochman *et al* 1992) and in one study amidated gastrins were found in only one of twelve human colorectal tumours (Van Solinge *et al* 1993). Much higher levels have been found in ovarian and bronchogenic tumours (Van Solinge *et al* 1993; Rehfeld *et al* 1989) and found in extracts of gastric antrum but sufficiently large samples of normal gastric antrum were not available for comparison as 'positive control' tissues in this study.

Most studies observed significantly greater amounts of progastrin in tumours relative to normal mucosa and the present findings support this. Overall, the few studies which have examined this subject have reported both quantitative and qualitative differences in gastrin peptide contents. This is possibly the result of differences in antibody specificities and sensitivities and differences in tumour characteristics such as stage, differentiation and cellular composition. Furthermore, when tissue is split into two aliquots (as in this study) the two pieces may not be entirely comparable and this has to be borne in mind when interpreting such studies.

There are several ways of studying expression of gastrin in colorectal tumours, each with its own relative merits. Study of gastrin mRNA expression by Northern analysis, and especially polymerase chain reaction, is very sensitive at detecting low levels of gene expression but the presence of mRNA may not necessarily mean that functional peptide is also present. Measurement of such peptides by radioimmunoassay of tissue extracts, as used here, is less sensitive but is semiquantitative and does allow study of the relative amounts of different processing products. Both of these methods fall down in one important respect in that they depend on tissue homogenisation and the cellular localisation of the gastrin-derived peptides therefore remains undetermined. The possibility that the peptides simply originate from specialised endocrine cells normally

present in sparse numbers in the colon cannot be excluded. Alternatively it is impossible to say whether all cells in a given tumour, or just a subclone, express gastrin. To answer these questions requires techniques such as in situ hybridisation or immunohistochemistry. Xerri *et al* reported that in situ hybridisation was suitable for demonstrating gastrin mRNA in antral mucosa but not sufficiently sensitive to allow detection of low level expression in colonic carcinomas (Xerri *et al* 1992). This is supported by the results of Northern analysis which has also been generally unsuccessful at detecting gastrin mRNA in the colon (Baldwin *et al* 1992; Finley *et al* 1993; Singh *et al* 1994). Immunohistochemical studies with the antibodies used in this study have not been performed but this is currently being considered. Finley *et al* found gastrin and progastrin to be localised to scattered endocrine cells in normal colon using commercially available region-specific antibodies (Finley *et al* 1993). In contrast the majority of malignant cells in most colorectal carcinomas stained positively for gastrin. This study is at odds with others which failed to detect significant amounts of G-17 in colonic neoplasms by radioimmunoassay (Van Solinge *et al* 1993) and highlights the need for complimentary experimental approaches to answer such questions.

As described above (Results), the assay methodology had to be modified to allow reliable detection of these peptides. Initial assay conditions had a lower limit of detection of approximately $3ng.L^{-1}$ (as used to measure plasma gastrin) and so gastrin-derived peptides may well have been present at levels below this detection limit. In support of this, when further tissue samples were analysed using assay conditions of ten-fold greater sensitivity, progastrin-derived peptides were found in virtually all tumours and corresponding normal mucosa. Because of presumed non-specific interference in the assay, however, the concentrations obtained were poorly reproducible when measured in doubling dilutions. For this reason these results have been excluded and only data for the remaining fifteen patients has been considered. The data are therefore insufficient to allow meaningful correlation of tumour gastrin content and circulating plasma gastrin levels in individual patients. In general, however, carboxyamidated gastrins were present in the colon at concentrations approximately twenty to fifty-fold lower than were found in plasma from both colorectal cancer and

control patients (Chapter 10). It is very unlikely that this source of amidated gastrin contributes significantly to circulating levels. Nothing is known about the plasma concentration of gastrin-processing intermediates in colorectal neoplasia.

No antibody specific for progastrin itself was available and the presence of progastrin has been assessed indirectly from the results obtained with antibody GP168 after trypsinisation of samples. Although an antibody to measure progastrin directly would have been ideal several other studies have utilised trypsin digestion as a reliable means of measuring progastrin (Kochman *et al* 1992; Lüttichau *et al* 1993; Van Solinge *et al* 1993). It is unclear why increased amounts of carboxyamidated gastrins were seen following treatment of tumours with trypsin as the antibody used (R98) does not measure progastrin. No difference at all was observed in normal mucosa and although the difference in tumour tissues reached statistical significance ($P = 0.03$), the actual difference in median concentrations was small (5.4pmol.g^{-1} before and 6.2pmol.g^{-1} after trypsin). The biological significance of this is uncertain and unlikely to be important. In contrast, the content of gastrin precursors and processing-intermediates measured by GP168 greatly increased in tumour extracts following trypsin digestion but not in samples of unaffected mucosa. These findings are in keeping with those of others (Kochman *et al* 1992; Van Solinge *et al* 1993) and suggest that colorectal tumours may synthesise preprogastrin at relatively higher rates than normal colon, but lack the ability to carry out full post-translational processing of the hormone.

It had been widely assumed that progastrin and glycine-extended intermediates lack biological activity and are unimportant. Unpublished results found gastrin precursors to have neither receptor binding activity nor growth stimulatory properties (quoted in Van Solinge *et al* 1993a). In 1994 several group presented preliminary evidence to contradict this finding. High concentrations ($> 10^{-6}$M) of glycine-extended gastrin (G-gly) were trophic to DLD-1 human colon cancer cells *in vitro* (Singh *et al* 1994). The gastrin/CCK-B receptor-positive AR42J cell line was also mitogenically stimulated by G-gly with increases in [^{3}H]thymidine uptake and ODC activity (Seva *et al* 1994). The evidence also suggested that G-gly acts through a separate as yet unidentified receptor mechanism. Others have also shown that an antibody to G-gly, but

not one to amidated gastrins, inhibited AR42J cell growth *in vitro* (Nègre *et al* 1994). Finally, nanomolar concentrations of G-gly stimulated gene expression of the H^+, K^+-ATPase alpha subunit in primary cultures of parietal cells (Kaise *et al* 1994), again possibly via a receptor subtype distinct from gastrin/CCK-B receptors. Gastrin processing intermediates may thus possess biological activity and their possible physiological and pathological relevance clearly merits reappraisal.

In conclusion, this study has found that the majority of colorectal tumours contained carboxyamidated gastrins at levels considerably higher than in some other studies. The levels were not significantly greater than found in matched samples of unaffected mucosa. Glycine-extended precursors were also present at comparable levels in both types of tissue but the data do suggest that tumours may contain significantly greater amounts of progastrin. Levels of peptides in tumours were significantly correlated with those in normal mucosa. Further experiments are needed to locate the precise cellular origin of these peptides as is evaluation of the possible trophic effects of progastrin and other processing intermediates on colorectal cancer cells.

CHAPTER 12

GASTRIN/CCK-B RECEPTORS IN COLORECTAL CARCINOMA

12.1 INTRODUCTION

Knowledge of gastrin/CCK-B receptors has been increasing rapidly since the cloning of the receptor gene from different tissues in several species (Wank *et al* 1992; Pisegna *et al* 1992; Kopin *et al* 1992). High-affinity gastrin/CCK-B binding sites have been identified on a number of established human colorectal cancer cell lines but in contrast little is known about such receptors in fresh human colonic tissues. Only one substantial study (Upp *et al* 1989), along with one brief report (Chicone *et al* 1989) and one abstract (Rae-Venter *et al* 1981), all from the same group, have demonstrated high-affinity gastrin receptors on crude membranes from fresh human colorectal tumours by radioligand binding. Others have used in vitro 'gastrin-responsiveness' to indicate the presence of receptors without demonstrating them directly (Watson *et al* 1989b). Only one negative study (which examined only two samples of normal human colonic mucosa) has been published (Kumamoto *et al* 1989) although publication bias may contribute to this.

Before the increasing number of new gastrin receptor antagonists can be considered as possible treatments for patients with colorectal cancer, the presence and characterisation of such receptors must be shown unequivocally. Also, selection of patients who might benefit from these drugs would depend on knowledge of their receptor status. The aim of this study was to establish a reliable and sensitive assay capable of detecting gastrin/CCK-B receptors on cell membranes and to apply this to primary human colorectal neoplasms. The well characterised rat pancreatic cancer cell line AR42J (Jessop and Hay 1980) possesses high-affinity CCK-A and gastrin/CCK-B receptors (Scemama *et al* 1987; Watson *et al* 1991) and was chosen as a 'positive control' for this study.

12.2 METHODS

12.2.1 Materials

Bovine serum albumin (BSA), calcium chloride ($CaCl_2$), dimethylsulphoxide (DMSO), human gastrin-17-I (met), N-[2-Hydroxyethyl]-piperazine-N'-[2-ethanesulphonic acid] (HEPES), magnesium chloride ($MgCl_2.6H_2O$), soya bean trypsin inhibitor (SBTI) and glycerol were all purchased from Sigma Chemical Co. (Poole, Dorset, UK). BCA protein reagent assay kit was purchased from Pierce Chemical Co. (Rockford, Illinois, USA). ^{125}I-tyr-human Gastrin-17 was purchased from NEN-Dupont (Stevenage, UK).

12.2.2 Gastrin Receptor Antagonists

L-364,718 and L-365,260 are highly selective and potent non-peptide antagonists capable of distinguishing CCK-A and CCK-B receptors, respectively (Lotti and Chang 1989; Chang *et al* 1986). Both were kindly provided by Dr B. Evans of Merck Sharp and Dohme (West Point, Pennsylvania, USA) and were dissolved in dimethylsulpoxide (DMSO). G-17 was dissolved in whole cell assay buffer and stored frozen at -20°C.

12.2.3 AR42J Cells

(i) Cell Culture

AR42J was kindly donated by Dr S. Watson, CRC Labs, University of Nottingham. Cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (Gibco, Paisley, Scotland) in a humidified atmosphere at 37°C containing 5% CO_2.

(ii) Preparation of Plasma Membranes

AR42J cells were harvested with 1mM EDTA. After centrifugation at 1000 g for ten minutes at 4°C, the pellet was resuspended in ice cold homogenisation buffer (50mM Hepes, 10mM $MgCl_2.6H_2O$, 1μM soya bean trypsin inhibitor, pH 7.0) and sonicated on ice in three 15 second bursts before centrifugation at 1000 g for ten minutes at 4°C. The supernatant was centrifuged at 30000 g for thirty minutes at 4°C in an ultracentrifuge. The resulting membrane pellet was resuspended in ice cold assay buffer and processed immediately for protein estimation using a Pierce protein assay kit. Aliquots used for membrane ligand binding were stored frozen at -70°C in assay buffer (plus 0.1% BSA and 15% glycerol).

(iii) Radioligand Binding

Either 1 x 10^6 cells.tube^{-1} of AR42J whole cells or 100μg protein.tube^{-1} of AR42J membranes were incubated in duplicate with 114pM ^{125}I-G-17 for 180 minutes at 22°C in a final volume of 0.4mls of assay buffer (50mM Hepes, 10mM $MgCl_2.6H_2O$, 0.1% BSA, pH 7.0) or membrane assay buffer (homogenisation buffer plus 0.1% BSA) for the measurement of total binding. The reaction was terminated by addition of 0.8mls of ice cold assay buffer and bound ^{125}I-G-17 was separated immediately by centrifugation at 13000 g for three minutes. The pellet was washed twice and then counted in a gamma counter. Non-specific binding was defined as binding in the presence of 119nM unlabelled G-17. Specific binding was obtained by subtracting the radioactivity of nonspecific binding from total binding and expressed as fmol G-17 bound.mg membrane protein^{-1}.

12.2.4 Human Colorectal Tumours

(i) Collection and Storage of Tumours

Paired samples of human colorectal tumours and macroscopically normal mucosa were obtained at surgical resection and collected as described in Chapter 11. Patient details are given in Table 12.1.

(ii) Preparation of Membranes

To assess whether the method of tissue preparation affected gastrin binding, tissues were prepared in one of two ways. Samples from three patients were mounted on ice in a cryostat (-30°C) from which multiple sections were cut and placed in tubes precooled on dry ice. Samples from a further seven patients were pulverised to a fine powder under liquid nitrogen. All tissue was stored at -70°C until assayed. It was then homogenised in 3mls of ice cold homogenisation buffer (50mM Hepes, 10mM $MgCl_2.6H_2O$, 15% glycerol, 0.1% soya bean trypsin inhibitor, 0.1% bacitracin, 0.1% phenylmethylsulphonyl fluoride, 0.1% bestatin, 1.5mM dithiothreitol and 100KIU.ml^{-1} aprotinin, pH 7.0) using a precooled polytron homogeniser. The homogenate was filtered through gauze mesh (100μm) and centrifuged at 400g for five minutes at 4°C. After the supernatant was centrifuged at 30000g for 60 minutes at 4°C, the resulting pellet was resuspended in 2.5mls ice cold homogenisation buffer and homogenised by hand using a hand held glass teflon homogeniser. Protein estimation was carried out as above and membranes stored (100-200μg.tube^{-1}) overnight at -70°C prior to radioligand binding.

(iii) Radioligand Binding

Membrane binding (100-200μg.tube^{-1}) was performed as for AR42J cell membranes although a range of concentrations of ^{125}I-G-17 was used (0.01 - 0.5nM). Reactions were carried out at 22°C for either 15 or 60 minutes and the assay buffer consisted of homogenisation buffer plus 0.1% BSA. Assays were performed in duplicate.

Case No.	Initials	Age	Sex	Tumour site	Histology	Different-iation	Dukes' stage
932616	M.McL.	48	M	rectosigmoid	adenocarcinoma	moderate	C
372926	B.T.	44	M	sigmoid	mucinous adenocarcinoma	moderate	C
882211	P.S.	56	F	sigmoid	adenocarcinoma	poor	C
394247	G.McK.	71	M	caecum	adenocarcinoma	poor	D
649444	A.B.	84	F	rectosigmoid	adenocarcinoma	moderate	C
835887	H.B.	66	M	sigmoid	adenocarcinoma	moderate	D
502088	G.A.	67	F	rectum	adenocarcinoma	moderate	B
322234	J.A.	70	M	rectum	adenocarcinoma	moderate	B
440457	T.W.	63	M	rectum	adenocarcinoma	moderate	B
926357	E.L.	56	F	sigmoid	adenocarcinoma	poor	D

Table 12.1. Patient and tumour details.

12.3 RESULTS

12.3.1 Assay Optimisation Using AR42J Cells

(i) Effect of Cell Number and Incubation Conditions

Thorough optimisation of the gastrin/CCK-B receptor assay was carried out as part of another ongoing study into these receptors (data not shown) and only details of the final optimised assay are given here. Specific binding of ^{125}I-G-17 to both whole cells and membranes increased linearly over cell concentrations ranging from 2 x 10^5 to 1 x 10^6 cells.tube^{-1}. Optimum binding to cell membranes occurred with 100μg of membrane protein at which binding was less than 10% of total. In all subsequent experiments a concentration of 1 x 10^6 cells.tube^{-1} or 100μg protein.tube^{-1} was used.

Specific binding of ^{125}I-G-17 to AR42J cells reached a maximum after 180 minutes incubation and addition of 1μM unlabelled G-17 into the incubation medium at this time (when the reaction was at steady state) caused dissociation of bound radioactivity.

For whole cells optimum binding occurred between pH 6.5 and 7.0 and decreased thereafter. The pH optimum for AR42J cell membranes was 7.0. These were therefore chosen for subsequent assays.

Maximum binding of gastrin to AR42J cells occurred at 22°C after incubation for 180 minutes. At 4°C binding was also increased compared to that seen at 37°C but not to the same extent as at 22°C. At 37°C binding decreased possibly as a result of proteolytic degradation of radiolabel and this was almost complete after 180 minutes. Thus 22°C was selected as the most practical temperature and subsequent experiments were performed in an ambient temperature water bath. Figures 12.1 and 12.2 show binding curves for gastrin under these optimised conditions.

(ii) Scatchard Analysis

From Scatchard analysis of three separate AR42J cell experiments the apparaent dissociation constant (K_d) for gastrin was 4 x 10^{-10}M. Using membrane preparations the K_d for gastrin was 1 x 10^{-9}M.

(iii) Effect of Antagonists on Binding to AR42J Whole Cells and Membranes

Using the optimised conditions above both L365,260 and L364,718 inhibited binding of ^{125}I-G-17 to AR42J whole cells and membranes (Figures 12.1 and 12.2). In these two preparations L365,260 caused half maximal inhibition (IC_{50}) at 0.9 x 10^{-8}M and 2.0 x 10^{-8}M, respectively. L364,718 was less potent in both preparations with IC_{50}'s of 2.5 x 10^{-7}M and 2.0 x 10^{-7}M, respectively.

12.3.2 Gastrin Binding in Human Colorectal Tumours

Details of patients and tumours are given in Table 12.1. Using concentrations of ^{125}I-G-17 ranging from 0.05 - 0.5nM (18 000 - 170 000 total counts added, approximately), little or no specific binding was observed in samples of either normal mucosa or colorectal carcinomas from any of ten patients. The amount of membrane protein used (100 or 200 μg) made no difference nor did the method of tissue preparation (pulverising under liquid nitrogen or cryostat sectioning). Binding data are shown in Table 12.2 and Figures 12.3 - 12.12. The figures are shown as histograms depicting actual binding data to emphasise that most of the binding was non-specic. In general there was insufficient tissue to allow multipoint saturation experiments to be run. As little specific binding was found in any of the samples analysed from ten consecutive colorectal tumour patients, it was felt unlikely that receptor binding would be observed in further samples using this methodology. Tumours from a further forty patients were not therefore assayed and this is discussed in detail below.

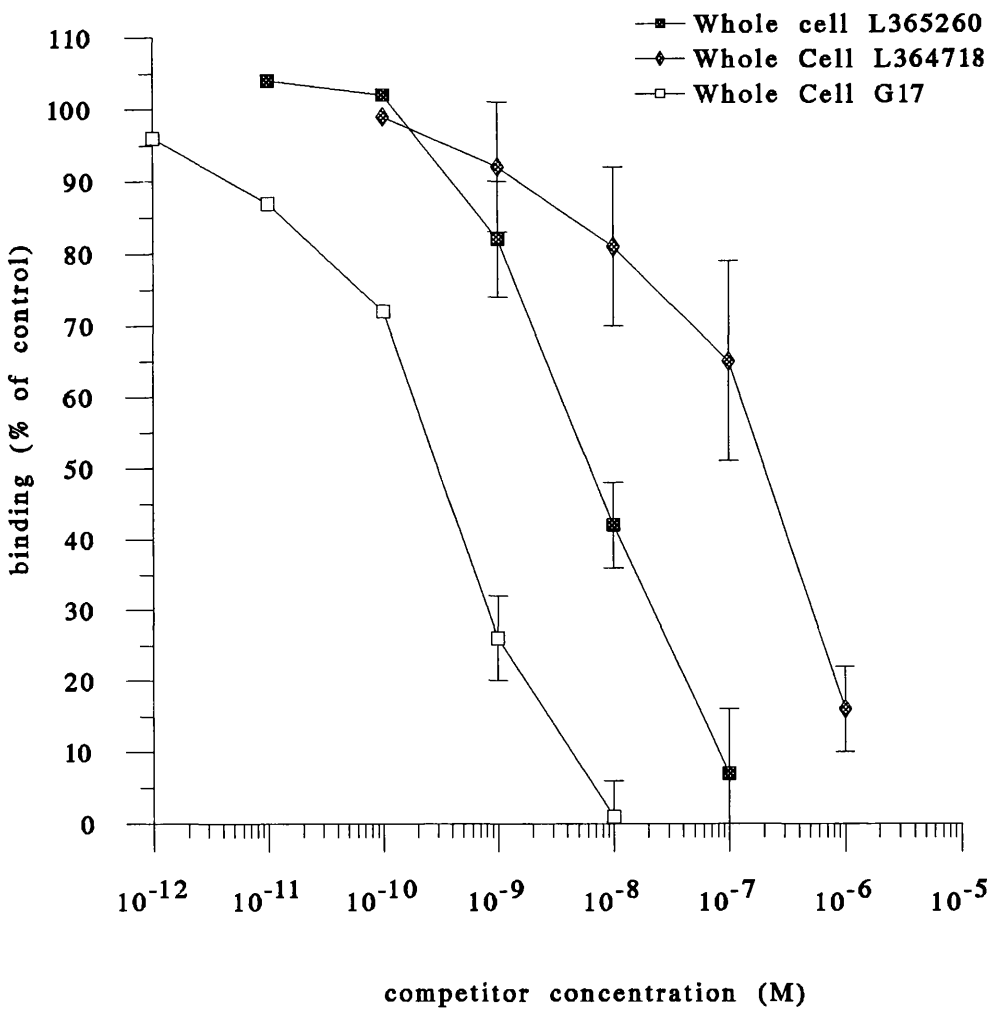


Figure 12.1. Displacement of [^{125}I]-G-17 from AR42J whole cells using G-17, L365,260 and L364,718.

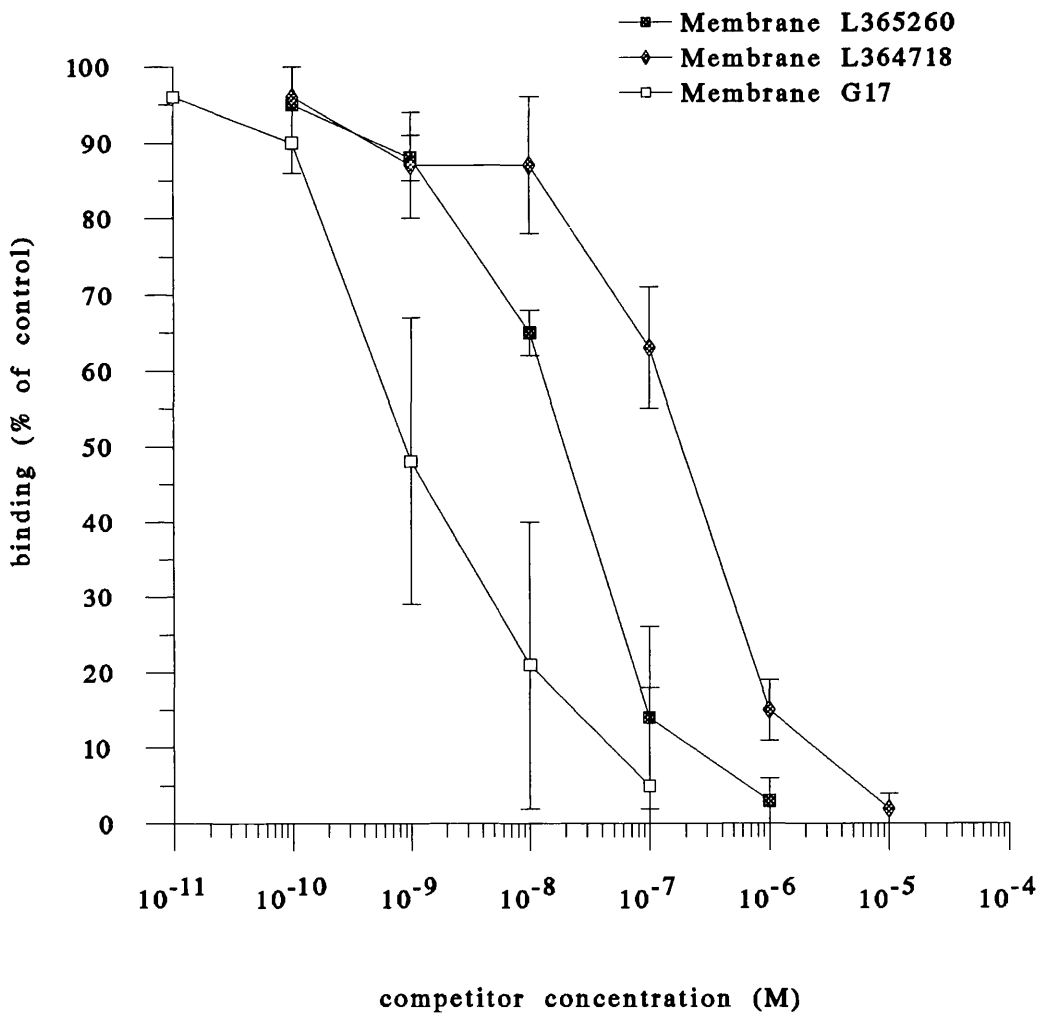


Figure 12.2. Displacement of [^{125}I]-G-17 from AR42J cell membranes using G-17, L365,260 and L364,718.

Case Number	Initials	Tissue	[^{125}I]-G-17 (nM)	Total Binding	Non-Specific Binding	Specific Binding
932616	M.McL.	normal	0.5	331	396	0
			0.25	145	130	15
			0.1	90	80	10
			0.05	<50	<50	0
932616	M.McL.	tumour	0.5	1433	1269	164
			0.25	497	433	64
			0.1	252	282	0
			0.05	131	128	3
372926	B.T.	normal	0.5	311	299	12
			0.25	222	189	33
			0.1	117	89	28
372926	B.T.	tumour	0.5	1512	1113	399
			0.25	740	736	4
			0.1	348	303	45
882211	P.S.	normal	0.5	272	279	0
			0.25	<50	<50	0
			0.1	155	82	73
			0.05	79	<50	0
882211	P.S.	tumour	0.5	633	697	0
			0.25	296	339	0
			0.1	170	131	39
			0.05	<50	89	0

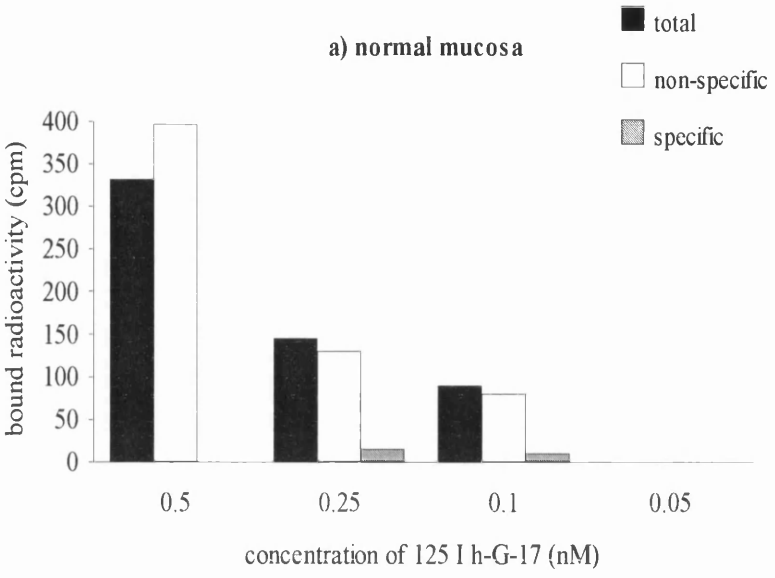
Table 12.2. Radioligand binding data (page 1 of 3).
Levels of binding indicate actual counts of radioactivity detected.

Case Number	Initials	Tissue	[^{125}I]-G-17 (nM)	Total Binding	Non-Specific Binding	Specific Binding
394247	G.McK.	normal	0.5	398	403	0
			0.25	224	198	26
			0.1	<50	<50	0
			0.05	<50	<50	0
394247	G.McK.	tumour	0.5	669	735	0
			0.25	434	366	68
			0.1	123	198	0
			0.05	89	103	0
649444	A.B.	normal	0.5	1894	1527	367
			0.25	1060	881	179
			0.1	478	410	68
649444	A.B.	tumour	0.5	4223	3929	294
			0.25	2453	2144	309
			0.1	896	870	26
835887	H.B.	normal	0.5	1104	791	313
			0.25	592	406	186
			0.1	225	162	63
835887	H.B.	tumour	0.5	2561	2015	546
			0.25	1406	1059	347
			0.1	458	443	15

Table 12.2. Radioligand binding data (page 2 of 3).
Levels of binding indicate actual counts of radioactivity detected.

Case Number	Initials	Tissue	[^{125}I]-G-17 (nM)	Total Binding	Non-Specific Binding	Specific Binding
502088	G.A.	normal	0.5	763	688	75
			0.25	326	285	41
			0.1	213	163	50
502088	G.A.	tumour	0.5	2673	2202	471
			0.25	1336	1235	101
			0.1	559	513	46
322234	J.A.	normal	0.5	474	462	12
			0.25	267	215	52
			0.1	131	109	22
322234	J.A.	tumour	0.5	883	729	154
			0.25	483	384	99
			0.1	235	183	52
440457	T.W.	normal	0.5	215	132	83
			0.25	161	121	40
			0.1	89	83	6
440457	T.W.	tumour	0.5	156	116	40
			0.25	60	53	7
			0.1	96	71	25
926357	E.L.	normal	0.5	233	172	61
			0.25	101	83	18
			0.1	84	66	18
926357	E.L.	tumour	0.5	1686	1567	119
			0.25	882	823	59
			0.1	423	292	131

Table 12.2. Radioligand binding data (page 3 of 3).
Levels of binding indicate actual counts of radioactivity detected.



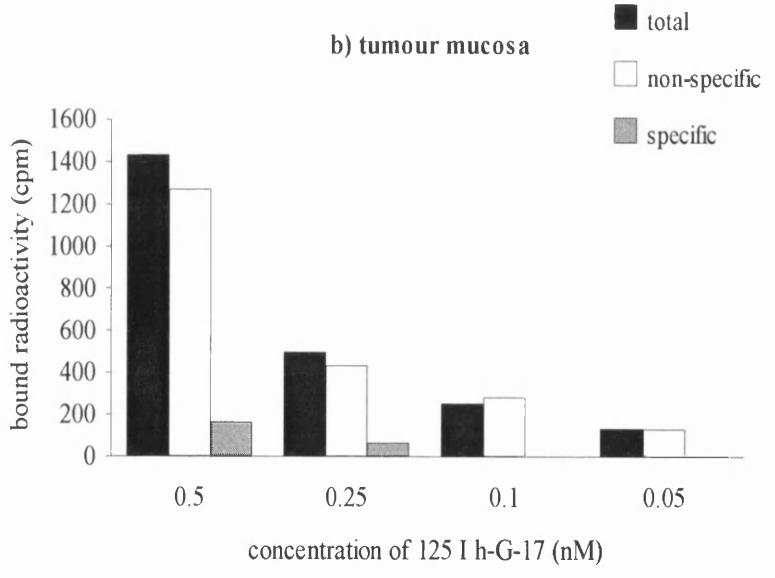
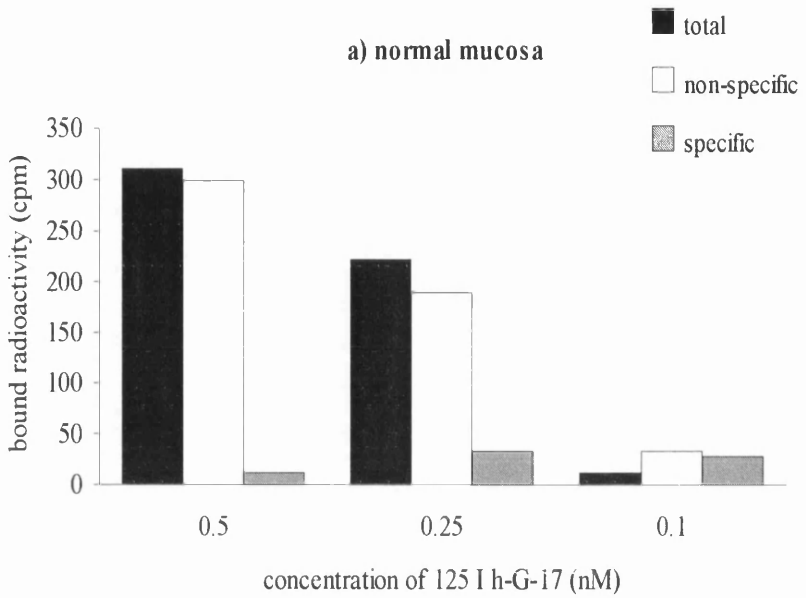


Figure 12.3. Radioligand binding of [^{125}I]-G-17 to a) normal mucosa and b) tumour mucosa from patient M.McL.. cpm = counts per minute.



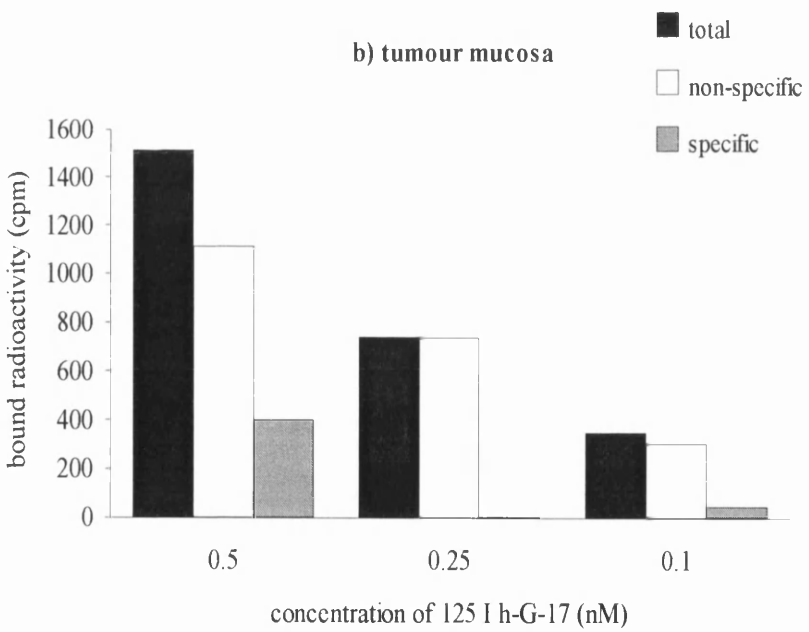
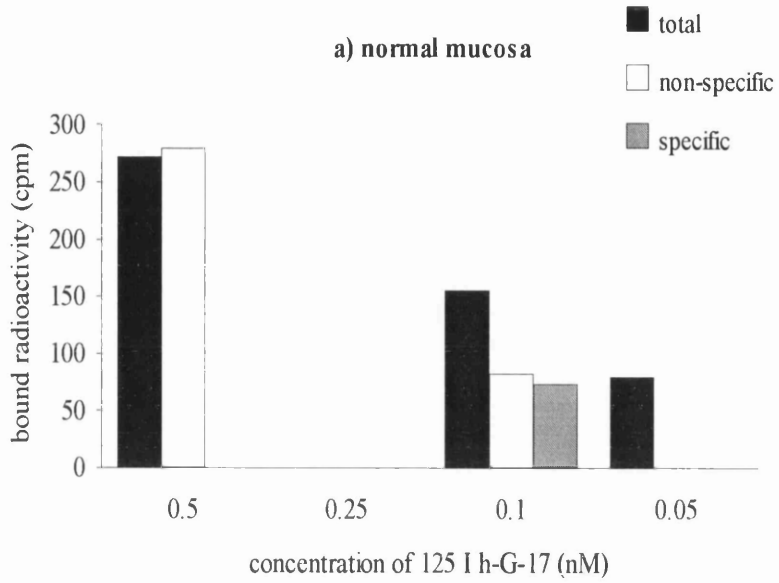


Figure 12.4. Radioligand binding of [^{125}I]-G-17 to a) normal mucosa and b) tumour mucosa from patient B.T.. cpm = counts per minute.



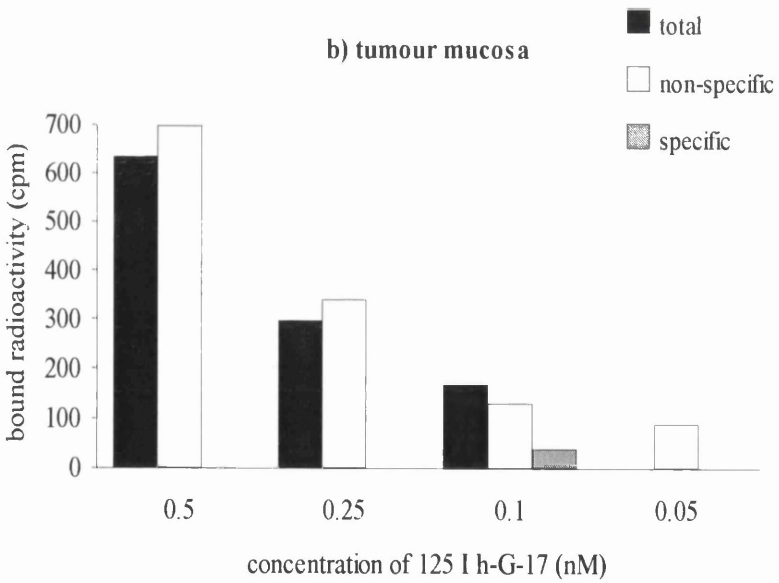
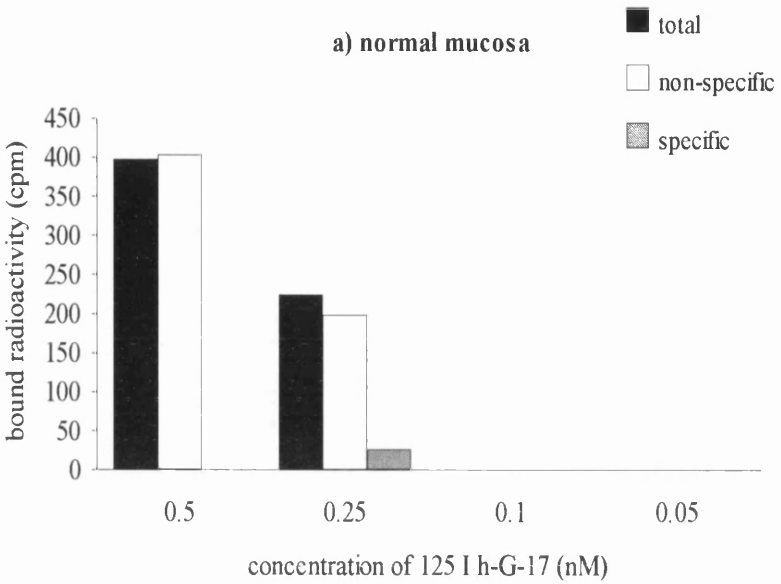


Figure 12.5. Radioligand binding of [^{125}I]-G-17 to a) normal mucosa and b) tumour mucosa from patient P.S.. cpm = counts per minute.



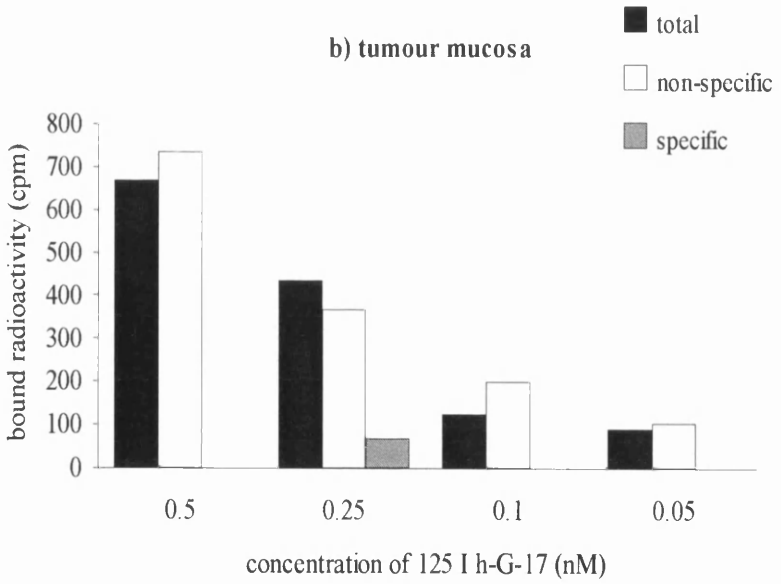
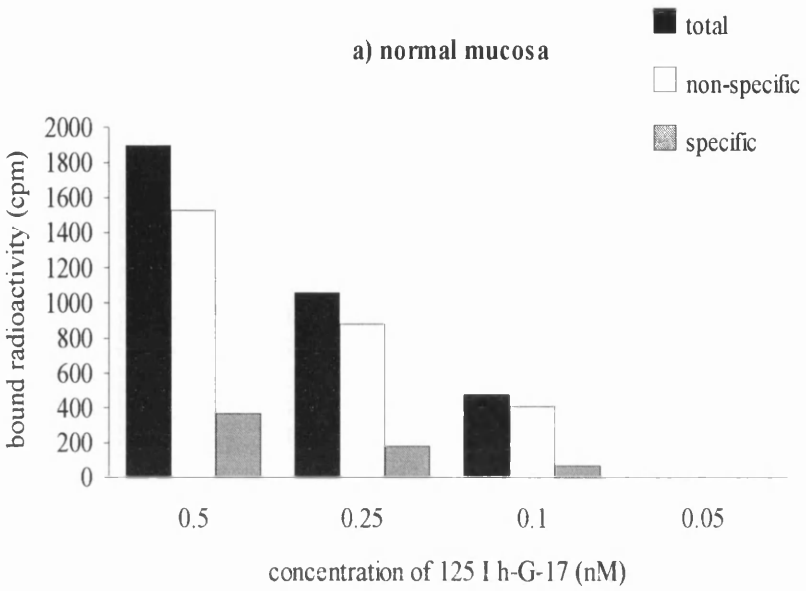


Figure 12.6. Radioligand binding of [^{125}I]-G-17 to a) normal mucosa and b) tumour mucosa from patient G.McK.. cpm = counts per minute.



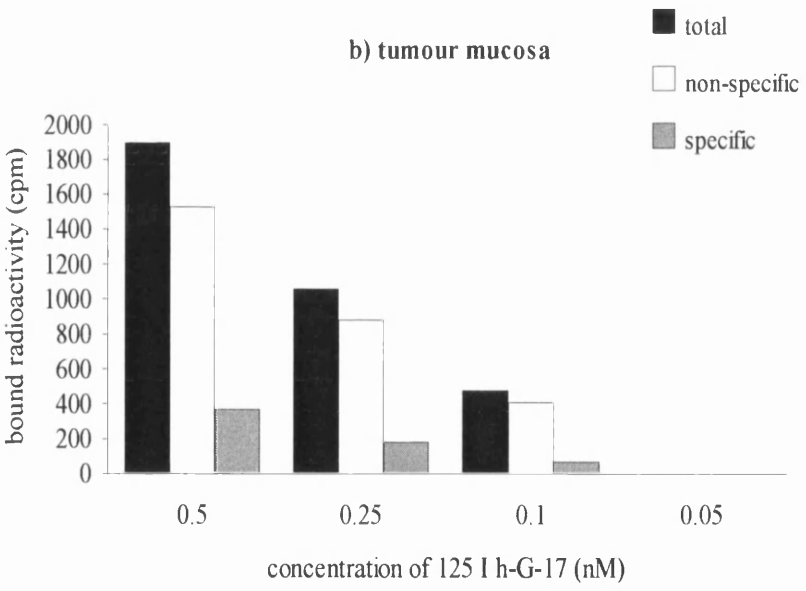
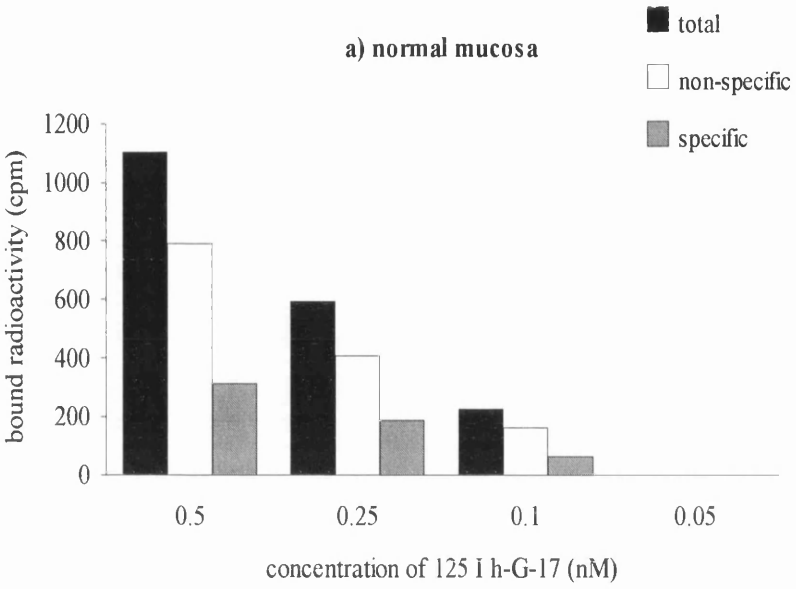


Figure 12.7. Radioligand binding of [^{125}I]-G-17 to a) normal mucosa and b) tumour mucosa from patient A.B.. cpm = counts per minute.



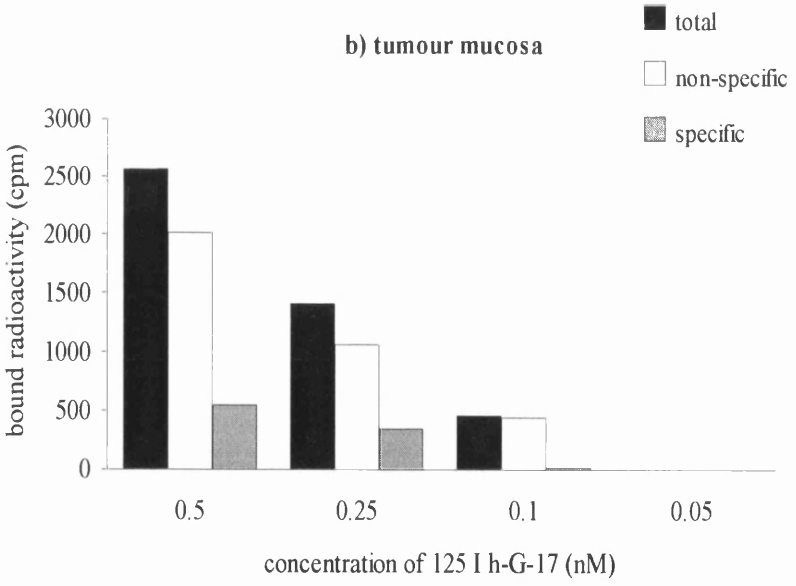
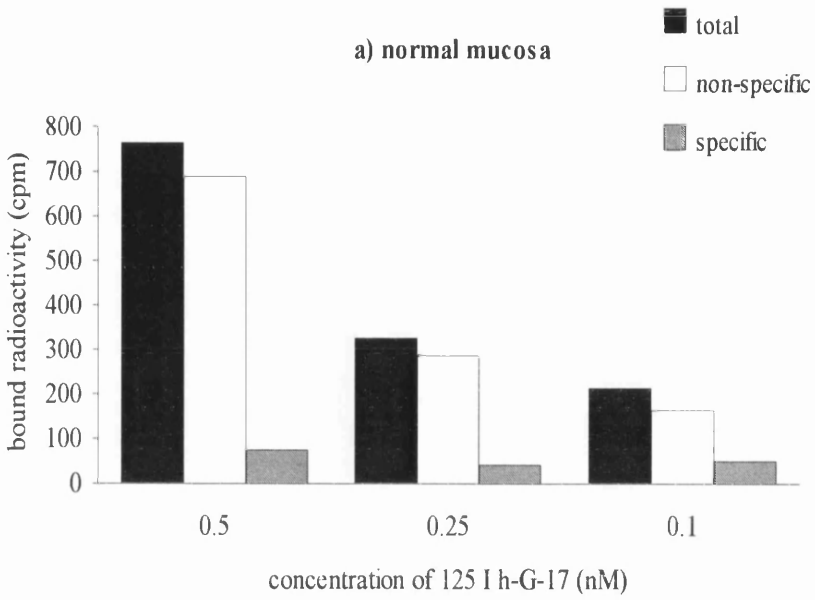


Figure 12.8. Radioligand binding of [^{125}I]-G-17 to a) normal mucosa and b) tumour mucosa from patient H.B.. cpm = counts per minute.



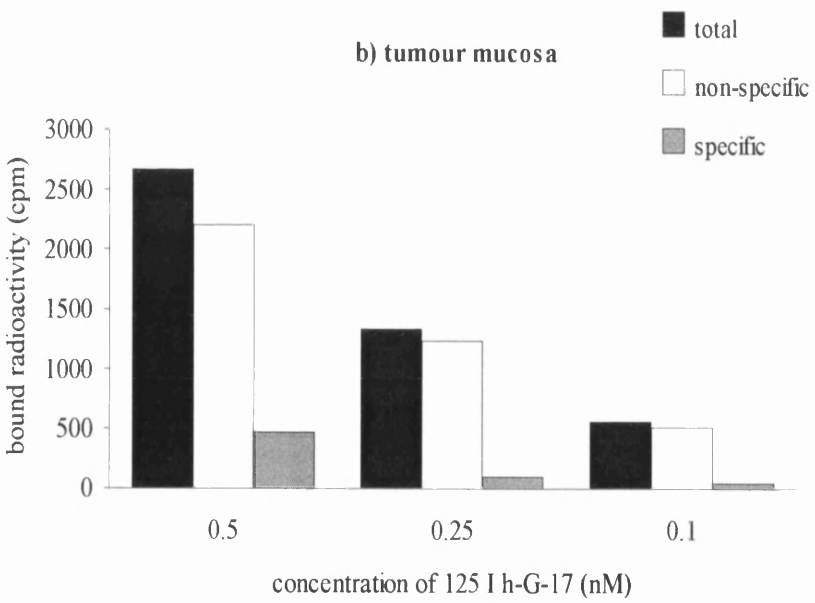
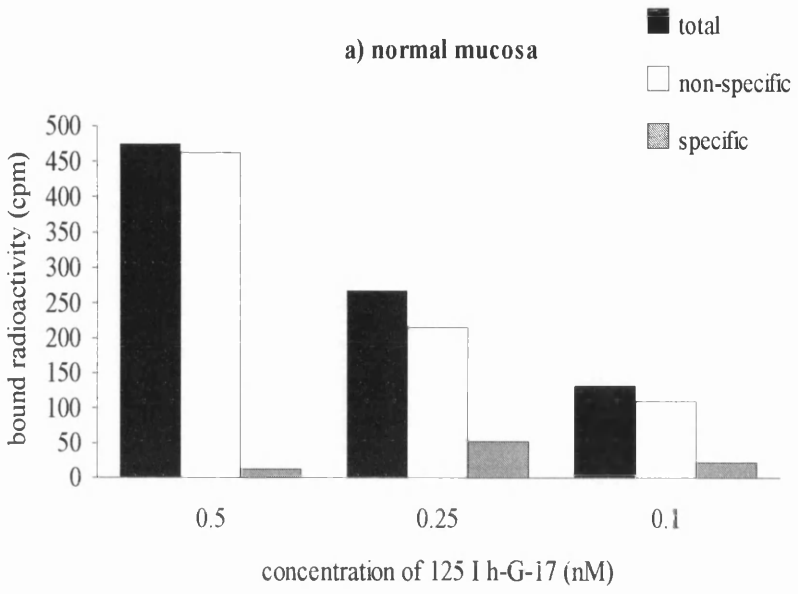


Figure 12.9. Radioligand binding of [^{125}I]-G-17 to a) normal mucosa and b) tumour mucosa from patient G.A.. cpm = counts per minute.



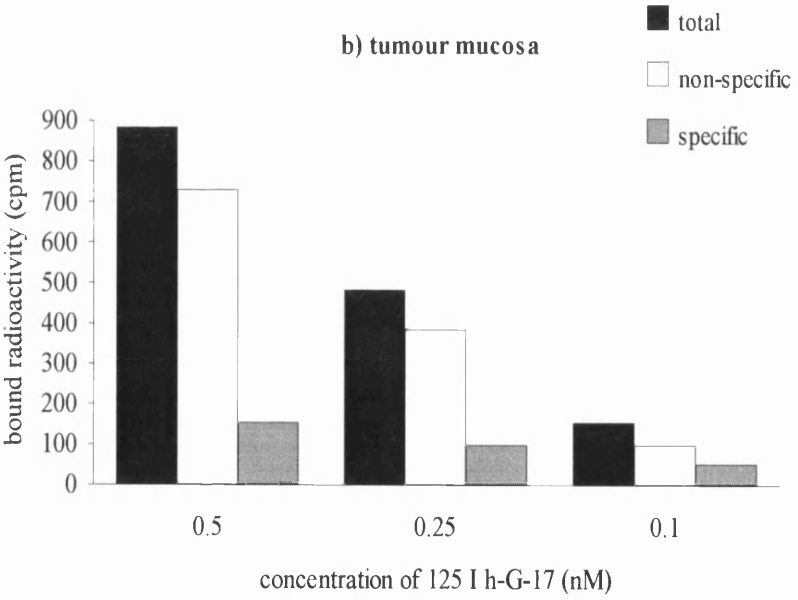
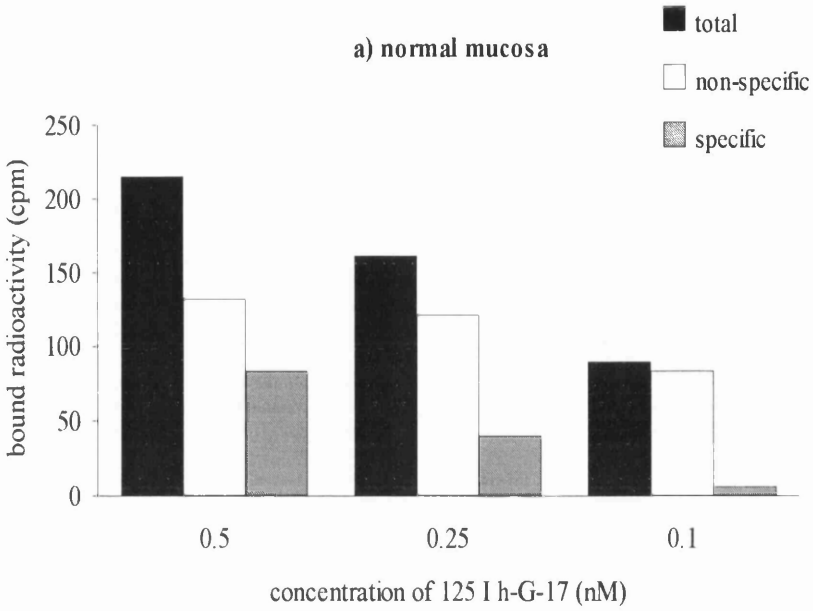


Figure 12.10. Radioligand binding of [^{125}I]-G-17 to a) normal mucosa and b) tumour mucosa from patient A.J.. cpm = counts per minute.



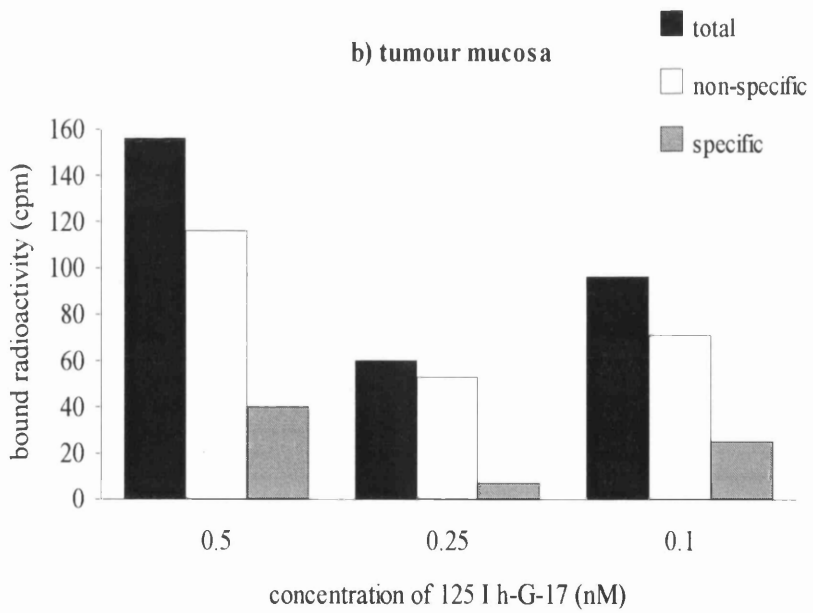
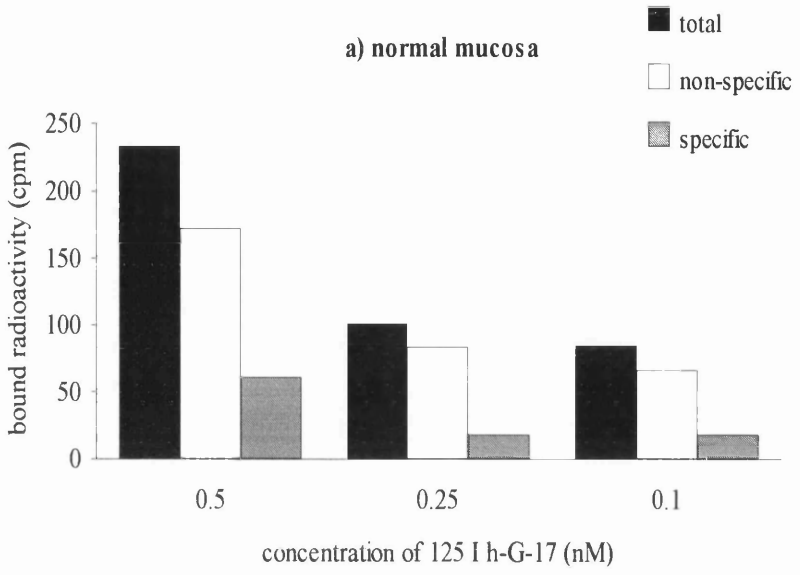


Figure 12.11. Radioligand binding of [^{125}I]-G-17 to a) normal mucosa and b) tumour mucosa from patient T.W.. cpm = counts per minute.



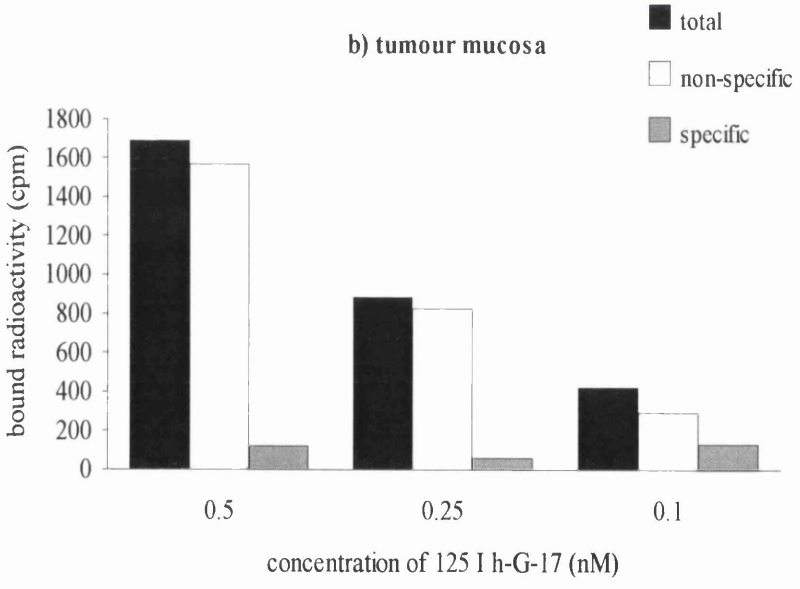


Figure 12.12. Radioligand binding of [^{125}I]-G-17 to a) normal mucosa and b) tumour mucosa from patient E.L.. cpm = counts per minute.

12.4 DISCUSSION

The aim of this study was to assess whether or not membranes from human colorectal cancers possessed measurable high-affinity gastrin/CCK-B receptors by radioligand binding. The technique itself was satisfactory as receptors were reliably and consistently measured on AR42J cells. In addition no significant loss of binding was observed in crude plasma membranes prepared from the cells. In contrast crude plasma membranes from samples of normal and malignant human colonic mucosa exhibited little or no specific binding when assayed in a similar way. There are a number of possible reasons why the human tumours gave such negative results.

Firstly, it is possible that typical gastrin/CCK-B receptors were not present on the colonic tissues examined. Although paired samples from only ten patients were analysed, the lack of specific binding made it unlikely that high-affinity binding sites would be found in further samples, at least using the current methodology. While gastrin/CCK-B receptors have been identified in normal and malignant colonic cells from a number of species (Singh *et al* 1985, 1986b; Guo *et al* 1990; Watson *et al* 1992), little is known about primary human colorectal tumours.

Upp *et al* found such receptors in approximately two-thirds of membranes from normal and malignant human colon but this is the only published detailed study which has measured gastrin/CCK-B receptor content directly in a substantial number of samples. In contrast Kumamoto reported no specific binding of gastrin in normal colonic mucosa from *two* patients (Kumamoto *et al* 1991). Other investigators have either used established human tumour cell lines (Frucht *et al* 1992) or have utilised in vitro 'gastrin-responsiveness' as an indirect measure of the presence of receptors in fresh human tumours (Watson *et al* 1988, 1989b). In these studies only a proportion of tumours or cell lines possessed receptors or responded to gastrin. Nevertheless, while only a proportion of colonic neoplasms may possess functional receptors, it seems statistically unlikely that all ten tumours analysed consecutively in this study would be

truly receptor negative. It is equally possible that gastrin/CCK-B receptors were present but were not detected by the protocol used.

The use of radioligand binding assays to detect peptide receptors in tissue preparations can be problematic with many points at which difficulties can arise. These have been described in detail elsewhere, both in general terms (Bylund & Yamamura 1990) and with reference to gastrin/CCK-B receptors (Kleveland and Waldum 1991). As the gastrin/CCK-B receptor may have a short half-life, the first possibility is that receptors were lost during tumour resection. The vascular supply to the region of bowel being resected is usually ligated early during surgery, resulting in an unavoidable period of ischaemia before actual resection and freezing of samples. It is unlikely that this could be responsible for loss of more or less *all* binding activity.

Secondly, the method of tissue storage may have resulted in loss of receptors although this seems unlikely as snap freezing in liquid nitrogen is widely used and this is the method used by the group from Texas (Upp *et al* 1989; Chicone *et al* 1989). They did not store their tumours in 20% sucrose as has been suggested by others as a means of preserving receptor structure.

Alternatively, either the method of membrane preparation or the radioligand binding protocol itself may have contributed to the negative results. In this study crude membranes were prepared from the colonic tissues in a manner similar to that of Upp *et al*, itself a standard technique. Again it is unlikely that all receptors would be lost during tissue break-up although some receptor damage may well occur. Pulverising tissue to powder under liquid nitrogen has been successfully used for studying a number of receptors in gastrointestinal tumours, including those for gastrin-releasing peptide (Preston *et al* 1993) and oestrogen (Singh *et al* 1993). To assess this further, however, tumours from three patients were prepared by cutting thick (20μm) sections using a cryostat at -30°C, followed by manual homogenisation. Again no specific binding was noted (data not shown). Thus, powdering of tumours under liquid nitrogen probably made little difference to receptor binding.

The binding assay itself was also very similar to that of Upp *et al*. Only minor modifications were made to incorporate the optimum conditions which had been

carefully determined for gastrin/CCK-B receptors in AR42J cell membranes. As such failure to detect binding is unlikely to result from choice of incubation conditions such as temperature, duration and pH, although further modifications to the assay conditions are still required. The protease inhibitors used should have been adequate to prevent proteolytic receptor degradation.

This study did differ from that of the Texas group who iodinated their own gastrin as opposed to using commercially iodinated gastrin-17 (NEN-Dupont), as used here. As excellent and reliable binding was noted in the AR42J cells, it would appear that biological activity of the [^{125}I]-labelled gastrin was unaffected.

If low concentrations of high-affinity binding sites were indeed present at concentrations below the level of detection of the assay, then steps such as increasing the concentrations of either tissue (above 200μg.tube^{-1}) or [^{125}I]-G-17 (above 0.5-1.0nM) may be worth exploring. It would also be interesting to perform the radioligand binding using membranes prepared from freshly resected, unfrozen tumours and this is currently planned.

In the widely quoted paper of Upp *et al* (1989) high affinity gastrin binding sites were present in 38 of 67 human colon cancers. The authors do not state what levels of total binding (as a percentage of total radioactivity added) were obtained or what proportion of this binding was specific. Close inspection of their data (especially Figure 1) suggests that maximum specific binding (5 fmoles) represented only 10% of total radiolabelled gastrin added. It is unclear what percentage of total binding this was. In general total binding in radioligand assays should be around 20-40% of that added and the higher the relative specific binding the better (certainly above 50% and ideally around 90%). Results should therefore be viewed in this context. In the present study total binding was no greater than 4% of added radioactivity, of which 10-30% was specific. These results are insufficient to allow one to say that high-affinity receptors are present but may be comparable to those of Upp. The low levels of specific binding seen in several patients (see Figures 12.7 - 12.11) could alternatively represent binding to low-affinity sites which have been noted by several groups. Unfortunately lack of tissue precluded enough data points to be performed for estimation of Kd's in these tumours.

Interestingly it is now increasingly recognised that receptors for CCK and gastrin may exist in multple affinity states (including a very low affinity state) and move between states by poorly understood mechanisms (Yu *et al* 1990; Talkad *et al* 1994a, 1994b; Huang *et al* 1994). Whether these low affinity receptors are of physiological relevance is currently unknown.

From the data in Table 12.2 it can be seen that total binding in tumours (expressed per mg of membrane protein) was generally two-three fold greater in tumours compared to corresponding normal mucosa from the same patient. Taken together the results suggest that receptors, possibly in low numbers, may be present at levels which are difficult to detect with the current assay. Further refinements to the assay protocol as discussed above are warranted before considering alternative methods of studying these receptors.

Given the apparent difficulties with radioligand binding, what alternatives are there ? Following the recent isolation and sequencing of the genes for CCK-A and CCK-B receptors from human and other species (Wank *et al* 1992; Pisegna *et al* 1992; Kopin *et al* 1992; Miyake *et al* 1994), one approach would be to examine receptor mRNA expression by in situ hybridisation or Northern analysis. Alternatively, immunohistochemical studies using specific antibodies may also be fruitful. To date only one such antibody, raised to canine parietal cells, is available (Mu *et al* 1987) but preliminary reports suggest that it may be useful for studies of human gastrointestinal tumours (Watson *et al* 1993). Hopefully further antibodies will become available in the near future. These approaches have the advantage of giving information about receptor distribution at the cellular level but are poorly quantitative and do not allow pharmacological receptor characterisation.

In summary, high affinity gastrin receptors were not found in samples of normal and malignant human colon, despite being measured reliably in a control cell line. Whether such receptors are truly absent, or present only as low affinity sites or whether limitations of the assay are responsible is unclear. Further refinements are required and then alternative strategies should be considered if high affinity receptors can still not be shown unequivocally.

PART 3

SUMMARY AND CONCLUSIONS

CHAPTER 13

SUMMARY, CONCLUSIONS AND FUTURE WORK

13.1 INTRODUCTION

The evidence for and against an important trophic role for gastrin in human colorectal neoplasia has been presented in this thesis and several aspects of the subject have been investigated. Firstly, a well established animal model of the disease was used to study the influence of omeprazole-induced hypergastrinaemia on experimental carcinogenesis. Then plasma gastrin levles in patients with establishe colorectal neoplasia were examined in a detailed study and the content of gastrin and its precursors in samples of normal and malignant colonic mucosa from these patients was also assessed. Finally, radioligand binding techniques were used to investigate the presence of gastrin/CCK-B receptors in membranes from human colorectal tumours.

13.2 OMEPRAZOLE AND AZOXYMETHANE STUDY

This study examined the effects of omeprazole-induced endogenous hypergastrinaemia on the subsequent development of azoxymethane-induced colorectal neoplasia in rats. Omeprazole was chosen because of the well documented elevations in circulating gastrin concentrations seen during therapy with this commonly prescribed drug. The azoxymethane model was chosen because of its similarities to human carcinogenesis and because it has been well characterised and widely used in recent years.

Despite having marked hypergastrinaemia throughout the study, significantly fewer rats in the omeprazole group developed tumours and had fewer tumours per rat. The reasons for this are unclear but omeprazole is a potent inducer of the cytochrome P450 enzymes responsible for the metabolism of numerous carcinogens. It is possible that omeprazole treatment altered hepatic and/or intestinal azoxymethane metabolism, rendering it less effective as a colonic carcinogen.

Future experiments are merited to investigate further the results of this study. In particular, experiments are planned to assess the effects of omeprazole when given only during the promotion phase of carcinogenesis i.e. *after* initiation with carcinogen

has been completed. In this way any effects of omeprazole on carcinogen metabolism would be controlled for. Secondly, it is important to try and dissociate the effects of omeprazole itself from those of the hypergastrinaemia it produces. This could be done either by using a group of antrectomised rats (low gastrin) or by treating a group of rats with a potent and specific gastrin receptor antagonist (for example, L365,260 or JMV 320) in addition to omeprazole. Both approaches, however, may introduce other potential compounding factors such as the effects of surgery on growth and weight gain and the possible independent effects of another drug on tumourigenesis. Finally, short term experiments to establish whether omeprazole acutely affects the metabolism of [^{14}C]-azoxymethane, by measuring exhaled [^{14}C]-azomethane, are feasible if expensive and may be worthwhile depending on the results of the other studies outlined above.

13.3 HUMAN PLASMA GASTRIN STUDY

This study was designed to address the unresolved issue of whether patients with colorectal neoplasia have elevated circulating gastrin concentrations relative to a control group of patients. Previous studies have often been poorly designed and none had controlled for the presence of HP infection, a common and important cause of hypergastrinaemia.

The study controlled for all known causes of hypergastrinaemia and measured both fasting and meal-stimulated plasma gastrin concentrations pre- and postoperatively. When studied in this manner, gastrin levels were similar in both tumour and control patients. In all but one case very high gastrins occurred in patients with positive gastric autoantibodies and were likely to result from undiagnosed atrophic gastritis or pernicious anaemia.

Furthermore, no fall in gastrin concentrations was seen following presumed curative resection. It was notable that five tumour patients with perioperative loss of HP infection had reductions in postoperative plasma gastrin. The results of previous studies should be reconsidered in the light of the important effects of HP infection on

plasma gastrin. The results of this study have shown conclusively that colorectal tumour patients do not have higher circulating gastrin concentrations than well-matched controls.

The design of the study, however, did not address the question of whether patients with pre-existing, chronic hypergastrinaemia are predisposed to development of colorectal neoplasia. In this study, the prevalence of HP infection was similar in the two groups and this has subsequently been supported by the results of two other small studies (Meier *et al* 1994; Justin *et al* 1994). Much larger, carefully designed epidemiological studies would be required to answer this question with certainty.

13.4 TUMOUR GASTRIN CONTENT STUDY

This study assessed whether measurable amounts of gastrin or its precursors are present in human colonic cancers and whether there are qualitative and/or quantitative differences between normal and malignant colon. The method used was chosen in preference to analysis of RNA content because RNA content may not reflect that of translated peptide(s). It was also used in preference to immunocytochemistry as the latter cannot give either quantitative results or reliable information about the content of various processing-intermediates of gastrin.

The study demonstrated the practical difficulties encountered in accurately measuring peptide levels in extracts of heterogeneous tissues. Once early technical problems of non-specific interference were resolved, the results showed that significant amounts of both bioactive, carboxyamidated gastrin and its processing-intermediates were present in both normal and malignant colon. Levels were similar in the two tissues although the results of trypsinisation suggest that tumours contain markedly more progastrin than corresponding normal mucosa. This is compatible with defective post-translational processing of gastrin in tumour cells although the lack of an antibody specific for progastrin itself prevents a definitive answer on this point. It would be

useful to extend this study using such an antibody and efforts to obtain one are currently being made.

As peptide concentrations were similar in both tissues it is unlikely that synthesis of gastrin peptides is a novel feature restricted to neoplastic colorectal epithelium. It does not detract, however, from the hypothesis that gastrin produced locally may be a relevant trophic factor for tumour cells at this site.

The main drawback of the study methodology was that precise cellular localisation of the site of gastrin production in the colon was not possible. Correlation of the current findings with those of immunocytochemical studies would be of value and are planned. Lastly, given the very recent preliminary reports that gastrin precursors may indeed possess functional activity, it is now timely to look more closely at what trophic roles these peptides may play in colorectal carcinogenesis.

13.5 GASTRIN/CCK-B RECEPTOR ASSAY

In this study the well characterised cell line AR42J was used as a 'positive control' to establish whether high affinity gastrin/CCK-B receptors could be detected by radioligand binding. This proved to be the case and no loss of binding was noted in crude membranes prepared from the cells. When a similar assay was applied to membranes from normal and malignant human colon, no convincing evidence for high affinity receptors was found and the possible reasons for this have been discussed. Despite following the methodology of Upp *et al* (1984) closely, significant high affinity binding was absent in ten consecutively analysed tumours. Further modifications to the assay protocol are necessary but it is unlikely that this will result in a great increase in binding above that which has been seen. Preliminary results from experiments using membranes prepared from freshly resected, unfrozen tumours are encouraging and suggest that a some tumours may exhibit high affinity receptors (J. McKenzie, personal communication).

As discussed in Chapter twelve, alternative methods to study these receptors are being considered. cDNA encoding rat and human CCK-B receptor genes has kindly been gifted by Dr. S. Wank, (Digestive Diseases Branch, National Institute of Health, USA) and plans are underway to develop an in situ hybridisation assay for gastrointestinal tumours.

Furthermore a panel of monoclonal antibodies specific for the gastrin/CCK-B receptor has recently been developed (Dr. S. Watson, personal communication) and it is hoped that these may soon be available for use in studies of human colorectal tumours. If satisfactory staining can be obtained in formalin-fixed, wax embedded tissues then it should be possible to analyse the colorectal tumours from the patients who participated in the studies described in Chapters eleven and twelve. Combined with radioligand binding studies of fresh cancers, these approaches may yield valuable information about whatever gastrin/CCK-B receptors may be present in normal and neoplastic colonic epithelium.

13.6 Conclusions

Reports of the role of gastrin as a trophic hormone for colonic epithelial cells continue to appear and yet its relevance to human colorectal neoplasia remains enigmatic. Resolution of this issue is important given the common and serious nature of the condition, the increasing longterm use of powerful acid-suppressing drugs with their attendant hypergastrinaemia and the development of new, potent and selective gastrin receptor antagonists as possible therapeutic agents.

The studies presented in this thesis have examined the role of gastrin in several ways. No convincing evidence supporting a simple trophic role in colorectal neoplasia was found. If such a relationship exists it is likely to be more subtle and complex than hitherto supposed. Further studies focusing on the autocrine/paracrine effects of gastrin peptides (especially progastrin and glycine-extended intermediates) are required and advantage needs to be taken of the recent cloning and sequencing of the gastrin/CCK-B

receptor genes. Together these approaches may define more closely what relevance, if any, gastrin has for tumours arising from the colon and rectum.

APPENDICES

APPENDIX ONE

RECONSTITUTION OF AZOXYMETHANE

1. Prepare stock solution (100mg.ml^{-1}) by diluting 1000mg azoxymethane (=1ml) with 9mls 0.9% sodium chloride. This stock solution should be kept in a lightproof glass container and stored at 4°C for up to one month.

2. On the day of injection, further dilute azoxymethane 10-fold with 0.9% sodium chloride (i.e. 1ml AOM stock solution + 9ml NaCl) to give a working solution of 10mg.ml^{-1}. Working solution should be made fresh each day and any unused solution discarded.

3. For a dose of 10mg.kg^{-1} body weight, this is equal to 1ml.kg^{-1} thus making calculation of dosing volume easier. This is further facilitated by using graduated U100 1ml insulin syringes (Becton Dickinson, Oxford, UK) which allows doses to be drawn up easily and accurately.

SAFETY PRECAUTIONS

1. All handling of azoxymethane must be done only by authorised personnel, after appropriate COSHH forms have been completed, approved and signed by all involved.

2. All handling must be performed in an approved fume cupboard or laminar flow hood with independent exhaust ventilation.

3. The following should be worn when handling azoxymethane:

   a) Plastic safety glasses - ("ARCO 40/1", Arco Walker, Glasgow, UK).

b) Organic Vapour Respirator ("3M5651", Arco Walker, Glasgow, UK).

c) Disposable theatre gown.

d) Plastic apron.

e) Two pairs surgical gloves.

f) Disposable overshoes.

4. All contaminated and used instruments, syringes etc. should be kept inside the fume cupboard and left to soak in a plastic "Sharpsafe" bin containing copious amounts of a strong oxidising solution such as sodium hypochlorite or potassium permanganate in sulphuric acid. At the end of the experimental period the bin should be sealed, double-bagged and incinerated.

5. Animal bedding and faeces should be handled with full precautions (as above), double-bagged, sealed and incinerated without opening.

APPENDIX TWO

RECONSTITUTION OF OMEPRAZOLE

1. Molecular formula : $C_{17}H_{19}N_3O_3S$.

2. Molecular weight : 345.42.

3. Required dose : 40μmol.kg^{-1} day^{-1}.

4. Required dose volume : 5ml.kg^{-1} , to give dosage volumes ranging from 0.75ml (for a rat weighing 150g) to 2.5ml (for a rat weighing 500g).

5. Conversion : 1μmol = 2.76mg.

6. Required omeprazole concentration: 22.08mg.ml^{-1}.

7. Range of dose volumes given : 0.75 (150g) - 2.5ml (500g).

APPENDIX THREE

PREPARATION OF OMEPRAZOLE SUSPENSION FOR TOXICOLOGICAL STUDIES

Raw materials

Omeprazole, micronised

Hydroxypropylmethylcellulose 15000 cps (Merrell Dow Ltd., Uxbridge, UK).

Sodium bicarbonate

2M sodium hydroxide

Distilled water

Preparation

1. Prepare a 0.5% HPMC solution as follows:

2. heat 1/4 of the total amount of water to 80-90°C and disperse the HPMC powder in the hot water under rigorous stirring with an intensive mixer (e.g. Ultra Turrax). Continue mixing until all particles are wet and then add most of the remaining cold water and stir until the solution is smooth.

3. add sodium bicarbonate ($2mg.ml^{-1}$) and adjust pH to 9.0 with sodium hydroxide.

4. add water to final desired volume.

5. place omeprazole in a suitable vessel, wet with a small amount of the HPMC solution and add the remaining HPMC solution slowly while continuously stirring.

6. dispense into sterile glass or plastic containers for a maximum of five days usage.

7. stir the suspension thoroughly before use to ensure no sediment remains.

Storage and stability

Store deep frozen (below -20°C) for no more than 24 months.

Thaw the suspension in a refridgerator (2-8°C).

The buffered suspension is stable for one week in a refridgerator, during which time it can be handled for two hours per day at room temperature.

APPENDIX FOUR

[^{14}C]-UREA BREATH TEST

Summary

Fasting subjects are given 0.4 MBq [^{14}C]-labelled urea and breath samples collected in hyamine hydroxide/thymolphthalein indicator solution at 10 minute intervals for varying lengths of time. The [^{14}C]-CO_2 collected in the solution is expressed as body weight in kilogrammes x % administered dose of [^{14}C] in sample/mmol of collected x 100 - i.e. Kgm % dose/mmol CO_2 x 100.

Reagent List

1. Water for injection - 1 x 10 ml Vial.
2. Hyamine hydroxide (1 molar) solution in methanol.*
3. Ethanol* (Industrial quality, obtained from University).
4. Thymolphthalein crystals (BDH Chemicals, Lutterworth, Leicester, UK).
5. Emulsifier-safe scintillation fluid*
6. Anhydrous calcium chloride (BDH Chemicals, Lutterworth, Leicester, UK).
7. "Ensure Plus" - 200ml (Abbott Laboratories Ltd., Maidenhead, UK).

* stored in an inflammable liquid safe.

3) Protocol

The subject is fasted from 2200 hours the previous night and then weighed with indoor clothes and shoes on.

The subject is asked to clean their teeth without swallowing any water, discarding all rinsings into running water in the basin.

The subject is shown how to give a breath sample - this is the baseline.

The subject is given the test meal to drink.

The subject is given the test dose (0.4 MBq [^{14}C]urea in 0.3ml water in 25ml water), then two rinsings of 25ml water. Note time i.e. Time 0.

The subject gives breath samples every 10 minutes for 30 minutes.

4) Isotope preparation and calculation of results

1. The dose is added into 25ml of water in a disposable paper cup and given to the subject to drink. The cup is flushed out twice with 25ml water and the subject also drinks this.

2. Two doses are diluted up to 50ml in a volumetric flask with ethyl alcohol and 2 x 3 x 1ml aliquots taken as standards and 1ml of 1M hyamine hydroxide/methyl alcohol added to each. Dilution factor = 50 *i.e.* Nett DPM x 50 = Counts/dose.

3. 2ml of indicator solution is used as a "machine negative [^{14}C]-CO_2 background" and unlabelled air is exhaled through this until the hyamine hydroxide is neutralised i.e. the blue colour disappears completely.

4. 2ml aliquots of indicator solution are used in the breath test for the subjects. They exhale through a kit containing calcium chloride until the hyamine hydroxide is neutralised *i.e.* the blue colour disappears completely.

5. Background, standards and samples have 10ml of emulsifier-safe scintillation fluid added into each. They are capped, mixed and the outsides wiped with an alcohol swab.

6. Samples are then counted on a liquid scintillation counter which has a [^{14}C] quench correction curve.

7. The subject's weight is incorporated into the calculation to correct for endogenous CO_2.

8. Calculation:

$$\frac{\text{Sample Nett DPM x Subject Weight in Kgm x 100 x 100}}{\text{Given dose DPM}}$$

$$= \frac{\text{Kgm \% dose/mmol } CO_2 \text{ x 100 excreted}}{}$$

9. The results are plotted on a graph of time (minutes) versus Kgm % dose/mmol CO_2 x 100 excreted.

APPENDIX FIVE

RADIOLIGAND BINDING ASSAY FOR HUMAN COLORECTAL TUMOURS

Assay volume	0.4ml
Membrane concentration	100μg.tube^{-1}
[^{125}I]-G-17 concentration	0.05 - 0.5nM
G-17 concentration	0.1 - 0.5μM
Time	15 minutes
Temperature	22°C
Homogenisation buffer	50mM HEPES 10mM $MgCl_2.6H_20$ 15% glycerol 0.1% soya bean trysin inhibitor 1.5mM dithiothreitol 0.1% bestatin 0.1% bacitracin 100KIU.ml^{-1} aprotinin pH 7.0
Assay buffer	as above plus 0.1% BSA

APPENDIX SIX

PUBLICATIONS AND PRESENTATIONS TO LEARNED SOCIETIES

Some of the work of this thesis has been presented to learned societies and published in either abstract or full form.

Presentations to learned societies

"Omeprazole-induced hypergastrinaemia and experimental colorectal carcinogenesis in rats." Surgical Research Society, London, January 1993.

"Omeprazole inhibits azoxymethane-induced colorectal carcinogenesis in rats." Caledonian Society of Gastroenterology, Glasgow, February 1993.

"Omeprazole inhibits azoxymethane-induced colorectal carcinogenesis in rats." American Gastroenterological Association, Boston, May 1993.*

"Fasting and meal-stimulated gastrin levels pre- and post- operatively in colorectal tumour patients". Caledonian Society of Gastroenterology, Dundee, November 1993.**

* Also presented as a poster at the British Society of Gastroenterology in Manchester, March 1993.

** Also presented as a poster at the British Society of Gastroenterology in Manchester, March 1994 and the American Gastroenterological Association, New Orleans, May 1994.

Publications

Penman ID, El-Omar E, McGregor JR, Hillan KJ, O'Dwyer PJ and McColl KEL. Omeprazole inhibits azoxymethane-induced colorectal carcinogenesis in rats. Gut 1993;34:1559-1565.

Penman ID, El-Omar E, McGregor JR, Hillan KJ, O'Dwyer PJ and McColl KEL. Omeprazole inhibits azoxymethane-induced colorectal carcinogenesis in rats. Gastroenterology, 1993; 104: A441 (abstract).

Penman ID, El-Omar E, McGregor JR, Hillan KJ, O'Dwyer PJ and McColl KEL. Omeprazole-induced hypergastrinaemia and experimental colorectal carcinogenesis in rats. Br. J. Surg., 1993; 80: 660 (abstract).

Penman ID, El-Omar E, Ardill JES, McGregor JR, Galloway DJ, O'Dwyer PJ and McColl KEL. Plasma gastrin concentrations are normal in patients with colorectal neoplasia and unaltered following tumour resection. Gastroenterology 1994;106:1263-1270.

Penman ID, El-Omar E, Ardill JES, McGregor JR, Galloway DJ, O'Dwyer PJ and McColl KEL. Plasma gastrin concentrations are normal in patients with colorectal neoplasia and unaltered following tumour resection. Gut 1994;35: S32 (abstract).

MacKenzie JF, Dorrian CA, **Penman ID**, Gerskowitch VP, McColl KEL. Development of an assay to detect gastrin receptors in gastrointestinal tumours. Gut 1994; 35: S73 (abstract).

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